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Regulation of the Nucleocytoplasmic Transport of HTLV-I RNA

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A thesis submitted in partial fulfilment of the
requirements of the Open University for the degree of
Doctor of Philosophy (Ph.D)

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This thesis is dedicated to the memory of
Dr. Sheila Lenman (G.P. St.Andrews, Scotland)

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Contents

1. Acknowledgements

2. Contents

3. List of Figures

4. Abstract

5. Introduction

Human T Cell Leukaemia Virus Type 1.....	1
HTLV-I linked Diseases.....	1
Organization of the HTLV-I Retroviral Genome.....	2
Organization of the Long Terminal Repeat.....	3
Gene Expression - Regulation by Tax and Rex.....	4
Expression of Two genes in <i>Cis</i> - Double Copy Vectors.....	6
Posttranscriptional Regulation in Human Immunodeficiency Virus Type 1.....	7
Protein and RNA Transport through the Nuclear Pore Complex.....	9
<i>Cis</i> -acting Positive and Negative RNA Elements.....	10
Project aims.....	12

6. Materials..... 13

7. Methods

Growth of Bacterial Cultures.....	20
Electro-competent Bacteria Preparation.....	20
Electroporation of Competent Cells with Plasmid DNA.....	21
Plating out Bacteria on Agar plates.....	22
Small scale (TELT) Plasmid DNA Preparation.....	22
Restriction Enzyme (RE) Digestions.....	23
Large Scale (Midi) Plasmid DNA Preparation.....	23
Large Scale (Maxi) Plasmid DNA Preparation.....	24
Freezing Bacterial Stocks.....	25
Gel Electrophoresis of Plasmid DNA.....	25
Polyacrylamide Gel Electrophoresis of Plasmid DNA.....	26
Determination of DNA and RNA Concentrations.....	26
Purification of DNA Fragments.....	26
Blunting of RE digested DNA ends.....	28

Contents

Dephosphorylation of Plasmid DNA ends.....	28
Kinasing of DNA ends.....	29
Ligation of DNA Fragments.....	29
Sequencing of Plasmid DNA.....	30
Polymerase Chain Reactions.....	31
Site Directed Mutagenesis.....	32
Labelling of DNA Fragments.....	33
Preparation of RNA from Eukaryotic Cells.....	34
Total RNA.....	34
Messenger RNA.....	35
Northern Blotting of RNA and labelled Probe Hybridization.....	35
cDNA Synthesis for RT-PCR.....	36
Sterile Cell Culture Techniques and Precautions.....	37
Passaging of Adherent and Non-Adherent Cells.....	37
Freezing and Thawing of Cells into and out of Liquid Nitrogen.....	38
Calcium Phosphate Transfection of Adherent Cells.....	39
DEAE-Dextran Transfection of Non-Adherent Cells.....	39
Electroporation of Eukaryotic Cells.....	40
β -Galactosidase Expression Assays.....	40
Chloramphenicol Acetyltransferase Expression Assay.....	41
Fluorescence <i>in-situ</i> Hybridization.....	43

8. Results

Section A: Sub-cloning of HTLV-I based Constructs

Cloning of the LTR _{JAY} Vector.....	45
Cloning of the Full Length LTR ₇₅₅ Vector.....	47
Insertion of the PGK promoter-Neo Resistance Cassette - DC Vectors.....	49
PCR Cloning of the Chloamphenicol Acetyltransferase Reporter Gene.....	49
Optimizing levels of Tax and Rex <i>Trans</i> -activation.....	52
Removal of the MLV Extended Packaging Sequence.....	53

Section B: Presence of a CTE inside the MLV Extended Packaging Sequence

Re-introduction of Psi Fragments.....	56
PCR Sub-cloning of the SV40 Origin of Replication.....	61
Fluorescence <i>in-situ</i> Hybridization - Determination of CAT RNA Location.....	63
Mapping of the Psi 312 Fragment to the MLV Genome.....	66

Contents

Section C: Identification of a Novel <i>Cis</i> -repressive Sequence	
Presence of a second CRS - inside the 3'LTR.....	69
Narrowing the Search to the RxRE of the 3' LTR.....	71
Specific Removal of the Rex Response Element from the 3' LTR.....	72
Specific Insertion of the Rex Response Element.....	74
Fluorescence <i>in-situ</i> Hybridization.....	78
Section D: Overview of Sub-cloning (Flow diagrams for all DNA constructs)	
9. Discussion.....	86
10. Abbreviations.....	96
11. References.....	99

Figures

<u>Figure</u>	<u>Page</u>	<u>Title</u>
Introduction		
1	2	Genomic organization of HTLV-I
2	3	Organization of the Long Terminal Repeat (LTR)
3	5	Regulation of HTLV-I gene expression by tax and rex
4	7	Different strategies for expression of two genes from one vector
Results		
5	45	Cloning procedure for reconstruction of JAY vector
6	46	DNA sequence of J157 LTR
7	47	PCR construction of full length (755bp) 5' LTR
8	47	Cloning of the new LTR-psi fragment in place of the old one
9	48	Cloning of construct J225 (Two full length LTRs)
10	49	Insertion of the PGK promoter-Neo resistance gene cassette
11	50	Double copy (DC) and standard CAT expression vectors
12	51	CAT expression assay of DC and standard CAT vectors
13	52	Optimization of co-transfection
14	53	Deletion of the extended packaging (psi) sequence
15	54	CAT expression assay of full length and CRS-deleted constructs
16	56	Introduction of a Hind III site (SDM) to enable psi sequence removal
17	57	CAT assay comparing expression of SDM and non-SDM constructs
18	58	Hind III linker insertion into the psi sequence
19	59	CAT assay of psi fragment containing CAT vectors
20	60	Repetition of CAT assays using COS7 and Hela cell lines
21	61	PCR amplification and cloning of the SV40 origin of replication - ORI
22	62	Northern blot from COS cells transfected with psi312 construct
23	63	Fluorescence <i>in-situ</i> hybridization of β -gal expressing NIH 3t3 cells
24	64	FISH to optimize nuclear / cytoplasmic staining in COS cells
25	65	FISH analysis of COS cells transfected with psi fragment constructs
26	66	Quantitation of nuclear : cytoplasmic ratios for figure 25
27	66	Mapping of the psi312 fragment to the MLV genome
28	67	<i>In-vitro</i> transcription constructs
29	69	3' LTR-deleted construct - LTR ₇₅₅ .CAT.poly(A)
30	69	CAT assay to examine role of downstream NTR

Figures

<u>Figure</u>	<u>Page</u>	<u>Title</u>
Results		
31	70	5'CRS- and 3' LTR-deleted construct - LTR ₆₂₈ .CAT.poly(A)
32	71	CAT assay showing result of 3'LTR and/or 5'CRS deletions
33	72	Specific removal of the rex response element (RxRE)
34	73	Effect of the RxRE removal upon rex dependency and inducibility I
35	74	Effect of the RxRE removal upon rex dependency and inducibility II
36	75	PCR cloning and insertion of the RxRE
37	76	CAT assay comparing the different RxRE insertions and deletions
38	77	Result of RxRE insertion/deletion upon CAT assays in COS & Hela cells
39	78	FISH of transfected and non-transfected COS cells
40a	79	FISH of COS cells transfected with full length CAT construct J337
40b-g	80	As figure 40a for constructs J338, J343, J341, J294, J342 and J320A
41	81	Quantitation of figure 40 nuclear / cytoplasmic FISH staining
Discussion		
42	90	3' RxRE-deleted constructs showing different levels of rex inducibility
43	91	Proposed secondary structure of RNA encoded by the 3' LTR

Abstract

Human retroviruses exert a high level of control over their own gene expression as well as that of their host cell. In addition to the structural genes (*gag*, *pol* and *env*), expressed by all retroviruses, human retroviruses also encode additional regulatory genes. One regulatory gene promotes transcription of the viral genome and modulates the expression of specific cellular genes. The second gene (expressed by HIV and HTLV viruses) regulates viral expression posttranscriptionally by overcoming the effect of *cis*-acting negative elements present within the incompletely spliced mRNAs.

In the human T cell leukaemia virus type 1 (HTLV-I) retrovirus, the regulatory gene is known as *rex*. The binding of Rex protein to a sequence at the 3' end of viral mRNA, known as the Rex response element (RxRE) leads to removal of posttranscriptional repression. A sequence within the U5 region of the 5' long terminal repeat (LTR) has previously been described as exerting posttranscriptional repression. This element was termed a *cis*-repressive sequence (CRS).

This thesis describes the discovery of a novel *cis*-repressive sequence present within the 3' LTR of HTLV-I, termed the "downstream negative element" or DNE. Its function is investigated and compared to that of the known CRS. Each repressive element is shown to partially block the nucleocytoplasmic transport of LTR transcribed mRNAs. Presence of both elements is seen to completely block transport. Deletion of both elements completely removes Rex dependency.

Also described is the discovery of a *cis*-positive element within the murine leukaemia virus extended packaging sequence (ψ^+). The MLV ψ^+ is used to enhance the packaging efficiency of pseudotyped retroviruses. Presence of the MLV ψ^+ is shown to partially remove Rex dependency from CRS-containing mRNAs. The positive element is shown to be present within the 3' terminal 312 bp of the MLV ψ^+ and to remove Rex-dependency by activating the nucleocytoplasmic transport of ψ 312-containing mRNAs. This element was termed a constitutive transport element or "CTE", after showing the same mechanism of action as a small element from the Mason-Pfizer monkey virus (MPMV).

- **Human T Cell Leukaemia Virus Type 1 (HTLV-I)**

HTLV-I linked Diseases

Advances in cell culture technology following the discovery of Interleukin 2 (IL-2) made the long term culturing of human T cells possible (Morgan *et al.* 1976, Ruscetti *et al.* 1977). HTLV-I was isolated from cultures of lymphoma patients' T cells four years later (Poiesz 1980a+b, 1981; Yoshida *et al.* 1982a+b). The first human retrovirus had been discovered. Epidemiological evidence has shown a clear association between infection with the HTLV-I virus and the disease adult T cell leukaemia / Lymphoma (ATLL) (Robert-Guroff and Gallo 1983; Wong-Staal *et al.* 1983, Yoshida *et al.* 1984; Miyoshi *et al.* 1981, Yamamoto *et al.* 1982, Popovic *et al.* 1983; Schüpbach 1989).

HTLV-I is endemic in Southern Japan and the Caribbean, with some regions of Japan having as many as 16% of the local population infected. Clusters of HTLV-I associated malignancies have also been documented in Southeastern USA, Central and South America, the Middle East and India.

Following infection with HTLV-I there ensues a lengthy incubation or latency period of between 20 and 40 years before the onset of ATLL, which fewer than 1 % of HTLV-I infected individuals actually go on to develop. Once ATLL is diagnosed, the disease progresses rapidly, patients having a median survival time of less than 12 months. As well as acute ATLL, some patients have a subacute or chronic disease. The subacute phase is characterized by >5% abnormal T cells in the peripheral blood and normal lymphocyte count. Elevated lymphocyte count ($> 4 \times 10^9 / L$) and serum LDH levels up to 200% of normal mark the onset of chronic ATL. Acute and lymphoma type ATL have a poor prognosis. ATL lymphoma patients have fewer than 1% abnormal lymphocytes and no lymphocytosis together with histologically-proven lymphadenopathy. The remaining patients are characterized as acute ATL, usually having tumour lesions but not satisfying the other three requirements (Shimoyama *et al.* 1991). HTLV-I infection has also been associated with diseases of the central nervous system (CNS). Tropical spastic paraparesis (TSP) and HTLV-I associated myelopathy (HAM) may develop only a few years after infection with HTLV-I. Whereas ATL results in a monoclonal expansion of T cells, TSP/HAM patients have a polyclonal population with multiple integration sites within the host genome (Osame *et al.* 1987). TSP/HAM results in the demyelination of nervous tissue. HTLV-I could also be linked to unexplained facial nerve palsy, rhombencephalitis and transverse myelopathy (Bartholomew *et al.* 1987; Gessain *et al.* 1986; Roman *et al.* 1987; DeFreitas *et al.* 1987).

Organization of the HTLV-I Retroviral Genome

HTLV-I like all other retroviruses expresses the three structural genes *gag*, *pol* and *env*. The *gag* gene (encoding p19 matrix-, p24 capsid- and p15 nucleocapsid proteins) and *pol* gene (encoding the reverse transcriptase, integrase and RNase H enzymes) are expressed from the full length genomic transcript with the *env* gene requiring a single splicing event. The protease (*pro*) gene is situated between the *gag* and *pol* genes and is expressed via a ribosomal frameshift (-1). As well as these structural genes, HTLV-I also encodes five regulatory genes (p40*tax*, p27*rex*, p12^I, p30^{II} and p21^{III}). The Tax and Rex proteins are expressed from two different reading frames after a second splicing event which involves the use of a splice donor (SD₂) site just inside the *env* gene and a splice acceptor (SA₂) site inside the pX region. It has been shown that the p12^I and p30^{II} gene products can be made via cryptic splicing into bp 6383 (SA₃) and 6478 (SA₄), respectively. These genes were termed *rof* and *tof* (Ciminale *et al.* 1992, 1995). p21^{III} is transcribed from an AUG within the *rex* reading frame. It is normally not expressed and has been suggested to antagonize Rex function. More recently it has been shown that deletion of the pX region between SA₂ and SA₃ does not effect expression of *tax*, *rex* and *env* (Roithmann *et al.* 1994).

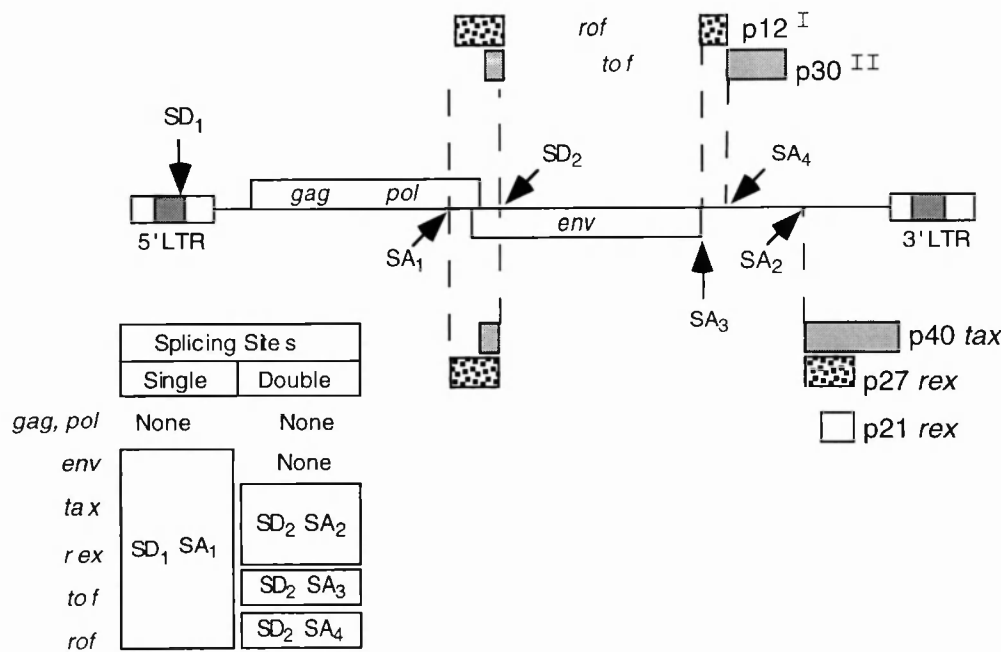


Figure 1. Genome organization of HTLV-I. Expression of Open Reading Frames (ORFs) from unspliced, single-spliced and double-spliced HTLV-I transcripts.

Packaging and formation of infectious virus particles requires that unspliced genomic transcripts and singly spliced *env* transcripts are able to leave the nucleus. A sequence mediating the packaging of HTLV-I genomic transcripts has not yet been identified as it is only recently that an infectious viral clone has been isolated (Derse *et al.* 1995, 1996; Zhao *et al.* 1995, 1996). The

original HTLV-I clone, which was sequenced by Seiki *et al.* (1983) was later shown to be non-infectious.

In contrast to the more simple retroviruses, human retroviruses have evolved to better regulate their gene expression once integrated in the host genome. The HTLV *tax* and *rex* gene products are responsible for *trans*-activation of the HTLV-I LTR and promotion of intron-containing structural gene expression, respectively.

• Organization of the Long Terminal Repeat (LTR)

HTLV (Human T cell Leukaemia Virus) and HIV (Human Immunodeficiency Virus) are the most intensively investigated retroviruses to date, not only in connection with their associated diseases, but also with relation to their regulation of viral gene expression.

The HTLV LTR shows well defined regulation at multiple levels. Although the LTR is present twice in the viral genome (once at each end) the regulatory elements are only active in either one LTR or the other.

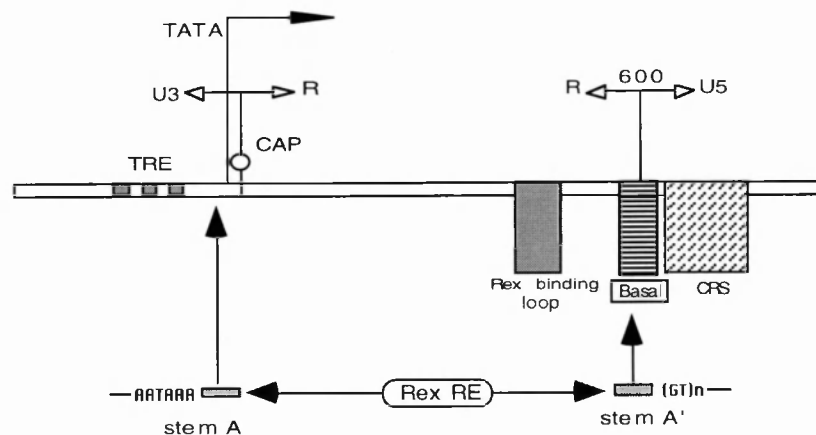


Figure 2: HTLV-I LTR elements required for regulation of gene expression.

Active in the 5' LTR

At the beginning of the U3-region is a 4bp consensus sequence which is cleaved specifically prior to integration of the provirus into the host chromosome. This is the upstream Integration site.

Inside the U3-region are situated the enhancer elements, the most important of which are known as the Tax response element (TRE). This consists of three 21bp repeats which can bind cAMP response element binding (CREB) proteins, following their interaction with the HTLV *trans*-activation protein Tax. Tax *trans*-activation of the LTR promoter results in a pulse of viral gene expression.

The viral promoter (TATA box) is situated just upstream of the U3/R boundary and is responsible for expression of all HTLV genes (*gag, pol, env, pro, tax* and *rex*). At the R/U5 boundary is a region

known as the CAS (*cis*-activating sequence). This is required for basal expression, in the absence of tax (Kashanchi *et al.* 1993). In the U5 region is the CRS (*cis*-repressive sequence) which appears to reduce expression from the LTR promoter. Part of this repression may be due to a block in transport of unspliced viral transcripts (*gag* and *pol*) - which encode this sequence within their 5' non-translated region (NTR) - from the nucleus to the cytoplasm (Seiki *et al.* 1990). At least in HTLV-II, the CRS inhibition is overcome by *rex* function (Black *et al.* 1991).

Active in the 3' LTR

The entire R region encodes a structure known as the Rex response element (RxRE). This high energy secondary structure is formed and stabilized by a main stem derived from sequences at the boundaries of the U3/R and R/U5 regions. This RNA secondary structure serves two purposes:

a. Formation of the polyadenylation motif

For correct polyadenylation, the poly A signal (AATAAA) and the GU-rich poly A site must be in close proximity. Formation of the RxRE (fig.2, stems A and A') brings these two regions, which are 255 nucleotides apart, in juxtaposition.

b. Formation of the Rex-binding motif (Bogard *et al.* 1992)

The HTLV regulatory protein - Rex - binds to a motif created in the RxRE. Rex binding has been linked to promotion of transport of unspliced (*gag* - *pol*) and single spliced (*env*) viral transcripts.

At the end of the U5 region is the second 4bp consensus sequence constituting the downstream proviral integration site.

• Gene Expression - Regulation by Tax and Rex

Upon entering the host cell, the two single-stranded RNA molecules that constitute the HTLV genome are reverse transcribed into DNA. This proviral DNA then integrates randomly into the host genome where its transcription can be activated by the action of Tax and the host's transcription machinery.

Tax *trans*-activates the HTLV-I promoter indirectly by interacting with and/or enhancing the binding of members of the CREB protein family (TREB 1,2,3,5,17;) to the imperfect 21bp repeats within the U3 enhancer region (Beimling *et al.* 1992, Suzuki *et al.* 1993). This region is known as the Tax response element (TRE). Tax has also been shown to *trans*-activate cellular genes such as interleukin 2 receptor alpha, granulocyte-macrophage colony-stimulating factor, *fos*, *jun*, *myc*, *sis* and MHC class I (Inoue *et al.* 1986, Maruyama *et al.* 1987, Alexandre and Verrier 1991, Fujii *et al.*

1991, Hirai *et al.* 1992, Himes *et al.* 1993). The diversity of Tax function upon cellular genes is partly due to its interaction with the NFkB/Rel family of proteins (Paul *et al.* 1993, Beraud *et al.* 1994, Lacoste *et al.* 1995).

Before strong Tax *trans*-activation can occur, Tax must be expressed. This could be achieved via two different mechanisms. Firstly, the U3 enhancer region contains binding sites for a variety of cellular factors. An increase in the binding of these factors would very quickly lead to a Tax-mediated amplification and a wave of HTLV-I gene expression. The alternative is that Tax is initially expressed from an internal promoter/enhancer element between the translation start sites (ATG) for *rex* and *tax* (Nosaka *et al.* 1993). Once Tax-induced transcription from the 5' LTR has been initiated, all transcripts are efficiently double-spliced, removing the two regions spanning from the middle of the R region (5' LTR) to the beginning of the *env* gene (splice 1) and from a splice donor just inside the *env* gene to a splice acceptor in the pX-region (splice 2). These transcripts, which are expressed independently of Rex function, encode the Tax and Rex proteins from two different reading frames. The export of unspliced and partially spliced transcripts is promoted by the Rex protein, which also functions to downregulate the expression of Tax and Rex genes, presumably through altering the efficiency of double splicing.

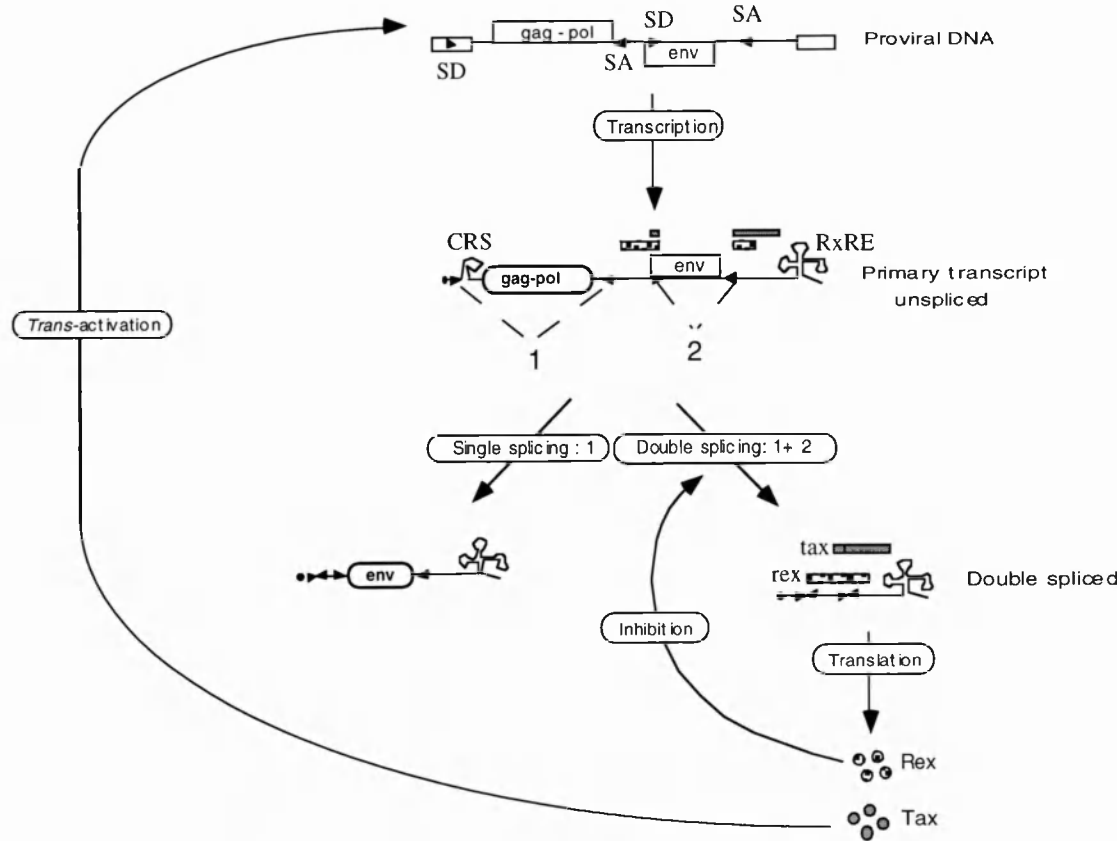


Figure 3. Regulation of HTLV-I gene expression by Tax and Rex.

The exact mechanism of Rex function has been difficult to dissect experimentally. Rex has been shown to bind to the highly organized Rex response element (RxRE) structure, present at the 3' end of all HTLV-I transcripts and lead to the posttranscriptional promotion of structural gene expression (Grassmann *et al.* 1991, Inoue *et al.* 1987). The observation that these incompletely spliced transcripts were *trans*-regulated by Rex only if they had a splice donor site suggested that Rex was functioning by interfering with the cellular splicing machinery (Seiki *et al.* 1988). Hidaka *et al.* (1988) showed that Rex expression led to an increase in incompletely spliced (*gag-pol* and *env*) gene expression and a suppression of completely spliced (*tax* and *rex*) gene expression. It was also shown that Rex expression led to an increase in unspliced RNA in the nucleus as well as in the cytoplasm (Inoue *et al.* 1991). The alternative hypothesis is that unspliced transcripts are retained in the nucleus due to the presence of *cis*-repressive sequences (CRS) and thus subjected to splicing and other nuclear processes. In this situation Rex binding would signal the export of the intron-containing transcripts. A CRS sequence has been identified within the HTLV-I 5' LTR U5 region (Seiki *et al.* 1990). In HTLV-II, Rex has been shown to be important in overcoming the CRS-mediated transport block, through binding the RxRE (Black *et al.* 1991).

• Expression of Two Genes in *Cis* - the Double Copy Vectors

There are a number of different strategies for expressing two or more genes in eukaryotic cells. The first strategy is to use an "internal promoter" (Wagner *et al.* 1985). For this a second promoter and second gene are cloned downstream of the first gene (fig. 4A). The second strategy is to introduce a 3' splice site (splice acceptor) between the two genes. This allows the two genes to be expressed from spliced and unspliced transcripts by the LTR promoter (fig. 4B). The third strategy involves the use of an "internal ribosome entry signal" (IRES) between the two genes (fig. 4C). This enables ribosomes to join the mRNA directly at the IRES instead of at the cap site (Jang *et al.* 1990a+b, Ghattas *et al.* 1991). Internal ribosome entry allows the expression of two genes from one promoter on the same mRNA molecule. All three strategies have been shown to work, however, none of them work in all situations. The internal promoter vectors are often prone to promoter silencing (Emerman and Temin 1986, Nakajima *et al.* 1993), meaning that the upstream promoter becomes more active than the downstream one, in some cases the downstream gene is no longer expressed. The splicing vectors do not always work because for efficient expression of both genes it is important to have inefficient splicing so that the first gene has a chance of being expressed. Because the efficiency of splicing not only depends on the sequence of the 3' splice signal but also upon signals present within the intron as well as the size of the intron itself, it has been difficult to design splice vectors that express both genes efficiently (Dougherty and Temin 1986). The IRES vectors offer a very attractive mechanism for expressing two genes, however, for reasons unknown the first gene is usually expressed preferentially.

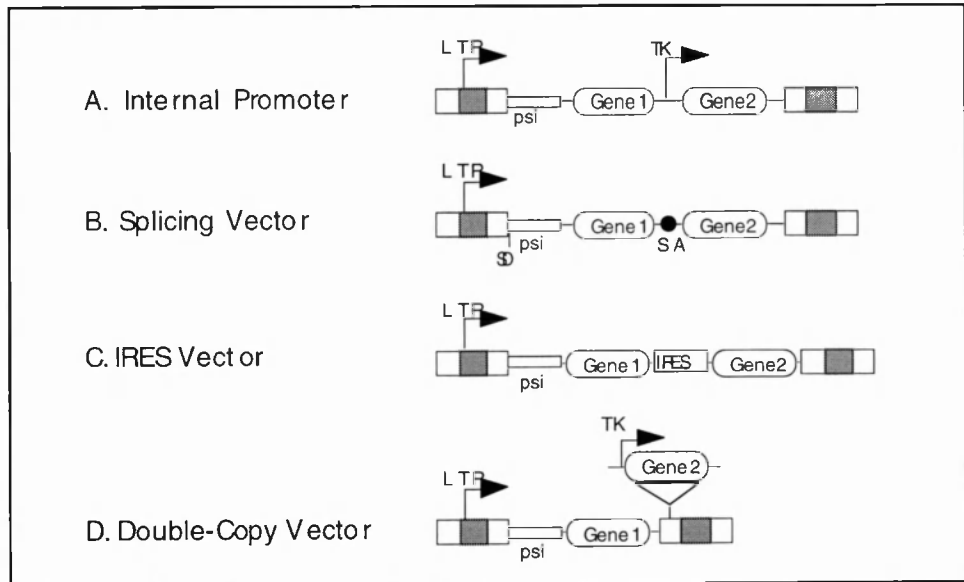


Figure 4. Different strategies for expression of two genes from a retroviral vector.

The double-copy (DC) vector was initially designed for higher expression of a single gene. Instead of cloning a gene between the two LTRs, a gene was cloned (with its own promoter) inside the U3 region of the 3' LTR. During reverse transcription of the viral genome, the U3 region of the 3' LTR is copied over to the 5' LTR. The resulting integrated proviral genome thus contains two copies of the inserted gene, one inside each of the LTRs. Stuhlmann *et al.* (1989) used a DC vector to express the dihydrofolate reductase (DHFR) gene. The development of high titer infectious virus showed that the U3 region insertions did not significantly interfere with viral replication. Hantzopoulos *et al.* (1989) also used an MLV based DC vector. This was used to express an ADA (adenosine deaminase) minigene. It was shown that insertion of the ADA minigene into the U3 region resulted in a 10-20 fold increase in the number of transcripts produced and human ADA isozyme synthesized in NIH 3T3 cells, compared to insertion of the minigene between the two LTRs. It was hoped that DC vectors would be more resistant to the gene silencing phenomenon observed following transduction of cells *in vitro* and *in vivo*.

• Posttranscriptional Regulation in Human Immunodeficiency Virus type 1

In 1984, a virus was isolated from a patient with lymphadenopathy syndrome (LAS) (Barré-Sinoussi *et al.* 1983) - a disease known to progress to acquired immune deficiency syndrome (AIDS). As initial sequence comparisons showed homologies to the HTLV viruses, the virus was initially termed HTLV-III. Following the complete sequencing of HTLV-I (Seiki *et al.* 1983) and characterization of a number of different HTLV-III isolates (Gallo *et al.* 1984; Levy *et al.* 1984; Popovic *et al.* 1984;

Sarnagadharan *et al.* 1984; Schüpbach *et al.* 1984a+b; Vilmer *et al.* 1984), HTLV-III was re-named Human Immunodeficiency Virus type 1 (HIV-1) (Coffin *et al.* 1986).

DNA sequencing of the first HTLV-I clone by Seiki *et al.* (1983) opened the door to a vast amount of information which later proved invaluable for workers in the HIV field. Although the two viruses share almost no sequence identity, the organization of the viral genomes and mechanisms of expression regulation are so similar that HTLV-I studies foreshadowed many important HIV discoveries made up until 1990.

The HTLV regulatory genes known as *tax* and *rex* (Kiyokawa *et al.* 1984,1985) were discovered first. The HIV equivalents *tat* and *rev* were then identified in 1985 (Arya *et al.*) and 1986 (Sodroski *et al.*), respectively.

HIV Rev was also shown to be required for HIV structural gene expression (Knight *et al.* 1987). In 1989 Hadzopoulou-Cladaras *et al.* and Malim *et al.* identified a Rev response element (RRE) within the *env* open reading frame. Rev binding to the RRE resulted in expression of the *gag* and *env* transcripts which had been shown to contain sequences that "contribute to low levels of mRNA accumulation". Interestingly, the HIV RRE is present only in structural gene mRNAs, whereas the HTLV-I RxRE is present at the end of all viral mRNAs. As in the case of Rex, HIV Rev has also been implicated in multiple regulation mechanisms. The observation that Rev expression also results in an increase in spliced mRNA and a decrease in unspliced mRNA hinted to an interaction with the cellular splicing machinery. It was shown that interaction of Rev with the U1snRNP (which binds 5' splice sites to initiate splicing) led to abrogation of splicing through blocking the association of U4/U6 and U5 snRNPs (Kjems *et al.* 1991, 1993).

The observation that cells not expressing Rev often contained incompletely spliced viral mRNAs in their cytosols hinted that Rev was able to release a blockage existing at the translational level. Arrigo *et al.* (1991) showed that Rev expression resulted in polysomal association of these incompletely spliced cytoplasmic mRNAs. Rev was shown to directly promote the nuclear export of RRE containing mRNAs from *Xenopus* oocyte nuclei (Fischer *et al.* 1994).

Following experiments which showed that Rex could substitute for Rev function (Rimsky *et al.* 1988) and that domain exchanges between Rex and Rev were functional (Hope *et al.* 1991), efforts were made to identify the repressive elements present in the HIV structural transcripts.

Cochrane *et al.* (1991) identified a 260 nt region of the HIV *pol* RNA which appeared to be preventing the unspliced mRNAs from being translated. Schwartz *et al.* (1992a) identified a 218 nt AU - rich region of the HIV *gag* gene which destabilized *gag-pol* transcripts in the absence of Rev protein. The same authors also demonstrated that through introduction of 28 point mutations into the destabilizing region, the negative effect upon expression was removed (Schwartz *et al.* 1992b). HIV *env* consists of two glycoproteins termed gp41 and gp120. The *env* heterodimer is termed gp160. In 1994 Brighty and Rosenberg identified a negative element within the RRE of gp41. Upon removal of the 240 bp RRE, resulting transcripts were no longer Rev-dependent. Also in

1994, Nasioulas *et al.* identified an additional CRS element, inside the HIV *env* gene, but this time within the gp120 coding region. They reported that this CRS was functional in human cells but not in *Drosophila* cells. This supported the observation that Rev is required for gp160- but not for gp120 expression in *Drosophila* cells (Ivey-hoyle and Rosenberg 1990). The gp120 CRS functions only in human cells, whereas the gp41 CRS (RRE) is thus functional in both human and *Drosophila* cells. This was the first evidence that a CRS element could be cell type specific.

Taken together, there is evidence that both HIV Rev and HTLV Rex are able to influence the expression of specific RNA species at different stages by overcoming the effect of various *cis*-repressive signals. It is possible that the different CRS elements are able to bind specific cellular factors which then inhibit the transcript's expression via different mechanisms.

• Protein and RNA Transport through the Nuclear Pore Complex (NPC)

It was once assumed that gene expression was controlled only at the transcription stage and that transcribed RNAs were able to diffuse passively through pores of the nuclear membrane into the cytoplasm. Examination of the nuclear pore complex (NPC) revealed that associated with the eight-multidomain pore are many protein factors. These were divided into two groups, the peripheral membrane proteins or "nucleoporins" and the integral NPC proteins. Three dimensional analysis suggests that only small molecules (<9 nm diameter) would be able to diffuse through the pore complex. Transport was shown to be an energy-dependent, carrier-mediated process (Zasloff 1983, Khanna-Gupta *et al.* 1989, Guddat *et al.* 1990, Dargemont *et al.* 1992, Dingwall and Laskey 1991).

Dividing HeLa cells must synthesize 10 million ribosomes each day. This process alone demands that each nuclear pore is able to transport 100 ribosomal proteins and 3 ribosomal subunits within 60 seconds (Görlich and Mattaj 1996). The energy for this transport process comes (at least in part) from the guanosine triphosphatase (GTPase) "Ran". Ran is associated with two other proteins which enable it to hydrolyse GTP and exchange the associated GDP for a new GTP molecule. Until now, the only Ran activating factor discovered has been the cytoplasmic RCC 1 gene product. The nuclear RCC 1 protein is responsible for guanine nucleotide exchange. The fact that the two Ran GTPase effector molecules appear to be separated by the nuclear membrane has led to some confusion. Various mechanisms have been proposed that explain the separate localizations. As shuttling of Ran itself through the NPC would be predicted to be an inefficient process, it has been proposed that proteins and RNPs could interact with different Ran molecules during translocation. It is also possible that the levels of effector protein required for transport do exist but that they are below the present detection limits.

The signals recognized by the transport mechanism are at the protein level. Nuclear import of proteins has been easier to study experimentally and thus nuclear localization signals (NLS) were

discovered first. More recently a motif was discovered which signals the rapid export of proteins from the nucleus. This nuclear export signal (NES) has been found within the HIV Rev protein (Fischer *et al.* 1995), the transcription factor TFIIIA (Fridell *et al.* 1996) and the protein kinase A inhibitor, PKI (Wen *et al.* 1995). A human factor hnRNP A1 was seen to shuttle between the nucleus and cytoplasm and be associated with polyadenylated RNA in both compartments. Upon closer examination, the protein was seen to contain both an NLS and an NES (Michael *et al.* 1995). It is very likely indeed that all RNA molecules are bound by proteins prior to export and that it is the protein that signals export and not the RNA. The best example of this is the specific binding of the HIV Rev protein to viral transcripts. Binding was shown to lead to transport of the otherwise retained RNA out of the nucleus (Cullen and Malim 1991). The "activation domain" of Rev was shown to contain an NES (Fischer *et al.* 1995).

As it has been shown that nuclear import and export of proteins through the NPC are linked - Ran GTPase most likely providing the energy for both - it was suspected that the basic mechanisms would be similar. NLS-containing proteins are not imported directly but are first bound by a two-subunit (α,β) receptor molecule termed "importin" (Imamoto *et al.* 1995). The NLS protein-importin α,β complex then associates with the outside of the NPC and is transported through, the importin subunits being recycled back to the cytoplasm (Görlich *et al.* 1995). By analogy, it was proposed that an NES receptor molecule would exist which could mediate the interaction with the NPC.

The two-hybrid system was used by three groups, to identify cellular proteins that were able to interact with the NES motif of Rev. Two groups found the same human protein termed "Rip" and "Rab" (Fritz *et al.* 1995; Bogerd *et al.* 1995), and the group using a yeast library identified a distantly related protein which they termed "Rip 1p" (Stutz *et al.* 1995). The Rip protein was then thought to be the Rev-NES receptor protein which mediates export of the Rev-RNA RNP. Subsequently, it was shown by Bevec *et al.* (1996) that eukaryotic initiation factor 5A (eIF-5A) is able to enhance Rev function through binding to the RRE-Rev complex. Two mutant eIF-5A proteins which were seen also to bind to the RRE-Rev complex, blocked Rev function through preventing the nuclear export of the RRE-Rev complex. Expression of the mutant eIF-5A proteins resulted in the inhibition of HIV replication in human CEM T cells. The authors concluded that eIF-5A is a cellular Rev-RRE binding factor, which either directly or via subsequent Rip/Rab binding, leads to the export of unspliced and incompletely spliced HIV mRNAs.

Xenopus oocyte injection of increasing amounts of a BSA-Rev NES fusion showed that export of 5s rRNA and U snRNAs but not mRNA, tRNAs and ribosomes could be competed. This suggests that although different classes of RNA are bound by specific proteins, the array of RNP-binding receptor proteins could be more limited (Fischer *et al.* 1995).

• *Cis*-acting Positive and Negative RNA Elements

As described earlier, the Rex and Rev proteins appear to be responsible for removing CRS-mediated expression blocks from specific viral transcripts. It has been proposed that CRS sequences function through binding cellular factors which are not processed by the cell's nuclear export system. Some non-retroviral expression systems also take advantage of such a posttranscriptional regulation. Influenza virus encodes an unspliced NS1 transcript which is freely transported to the cytoplasm where it is subsequently translated. The NS2 protein is expressed from the singly spliced form of the NS1 transcript. Initially both proteins are freely expressed. Expression of both NS1 and NS2 is then abrogated following the binding of the NS1 protein to a sequence within the NS2 mRNA. Both spliced and unspliced transcripts were seen to be trapped in the nucleus (Alonso *et al.* 1992). The fact that the unspliced NS1 transcript was allowed to leave the nucleus suggests that present within the transcript was a *cis*-positive element which perhaps via binding a cellular factor was able to activate the transcript's nuclear export. However, following binding of NS1 protein to its transcript, this hypothetical positive element's effect was no longer evident.

The U1 small nuclear RNA (snRNA), important for recognition of splicing signals and initiation of splicing when in its RNP form, was shown to encode two *cis*-elements which both act to promote the transcript's transport to the cytoplasm and subsequent expression (Terns *et al.* 1993). A 124 nucleotide stretch at the 5' end of the U1snRNA and a 3' terminal stem loop structure were shown individually to be capable of promoting the nuclear export of U6 snRNAs when inserted into the U6 coding region. U6 snRNA is normally retained in the nucleus. These experiments were performed via injection of specific snRNA into *Xenopus* oocyte nuclei.

Intron containing genes are usually more strongly expressed than "intronless" genes, presumably because the process of splicing stabilizes transcripts and promotes their passage on to the export system. One example of an "intron dependent" gene is the β -globin gene. If the β -globin introns are deleted, the resulting cDNA becomes trapped in the nucleus. The herpes simplex virus thymidine kinase (HSV-*tk*) has been shown to be "intron independent". As it does not require an intron for efficient expression it was hypothesized that a *cis*-acting positive element must be present in the HSV-*tk* transcript. This was tested by Liu and Mertz (1995) as they inserted fragments of the HSV-*tk* gene into an intron-deleted β -globin expression construct. It could be shown that a 119 nucleotide region (from 361 to 479) of the *tk* gene was capable of promoting the cytoplasmic accumulation of the intronless β -globin transcript. The authors concluded that the main effect of the 119 nt fragment was upon nucleocytoplasmic transport. The cellular factor found to bind to this element was the 68kD hnRNP L protein. The binding was shown to be highly specific and to depend upon the element's secondary structure as well as nucleotide sequence. This resembles the Rev / RRE and Rex / RxRE mechanisms of HIV and HTLV. In this case the regulatory protein is a cellular factor present in almost all cell types whose binding to defined RNA element

allows the transcript to leave the nucleus and be efficiently expressed. Hepatitis B virus (HBV) has also been described to contain a *cis*-acting positive element, reported to function similarly to a combination of Rev protein and the RRE (Huang and Liang 1993). A region just downstream of the *env* gene (nt 1200 to 1650) was shown to be essential for HBV gene expression and to function at the posttranscriptional level to inhibit weak splicing events and promote nuclear export of unspliced viral transcripts. When inserted between HIV splice sites, the element could inhibit splicing. However, the strong splicing of the β -globin pre-mRNA could not be inhibited following insertion of the element into the IVS2 β -globin intron. The authors propose that a cellular factor is able to bind this element and produce the same effect as HIV Rev. ie. The inhibition of weak splicing, the promotion of nucleocytoplasmic transport and subsequent translation.

Finally, Bray *et al.* (1994) showed that a region of the Mason Pfizer Monkey Virus (MPMV) when inserted into a Rev-dependent *gag* expression plasmid was sufficient to render the expression of *gag*, Rev independent. Although it has not yet been shown, binding of a cellular factor to the MPMV sequence could have overcome the transport repression mediated by a cellular CRS binding factor. The element identified by Bray *et al.* was termed a constitutive transport element (CTE). The authors suggest that a cellular pathway exists for the transport of intron containing transcripts, that are normally prevented from leaving the nucleus.

• Project aims

The initial aim of this project was to construct and test retroviral vectors based on HTLV-I. The strong posttranscriptional repression of HTLV-I LTR transcribed mRNAs together with the efficient removal of this repression upon the expression of the non-toxic HTLV-I Rex protein made HTLV-I based retroviral vectors attractive as a tool for the inducible expression of exogenous genes. As construction of retroviral vectors necessitates the removal of all structural and regulatory genes from between the LTRs as well as the introduction of "foreign" sequences such as the murine leukaemia virus packaging sequence and the gene(s) of interest, it is important to determine whether these changes lead to alterations in the pattern of LTR directed expression compared to the wild type virus.

As mentioned earlier, the CRS element within the 5' U5 region has been shown to be responsible for the posttranscriptional expression block and thus the requirement for the regulatory Rex protein. It was important to determine whether presence of this CRS was sufficient for production of Rex-dependent expression or whether other sequences were also required.

It is clear that Rex expression is required to release the CRS-mediated repression. However, as Rex and its HIV equivalent Rev have both been shown to enhance expression at various posttranscriptional levels, it was important to determine at which level the HTLV-I CRS was functioning.

• **Chemicals**

Ampicillin (Na salt)	Serva
Ammonium persulphate (APS)	Bio-rad
Bactoagar	Gibco
BES buffer	Serva
Bio-rad Protein Assay solution	BioRad
BSA	Sigma
Chloroquine	Sigma
DEAE Dextran	Sigma
DMSO	Fluka
EDTA	Merck
EGTA	Sigma
Ethidium Bromide	Sigma
Glycerol 86%	Roth
Inorganic salts were supplied by Merck and Roth	
Mineral oil	Serva
MOPS buffer	Merck
Organic solutions were supplied by Merck and Roth	
Serdolit ion-exchange beads	Serva
TEMED	BioRad
Triton X-100	Fluka
Ultrapure Urea	Roth
Yeast extract	Difco

• **Fine Biochemicals**

Acetyl Co.A	Sigma
¹⁴ C-Chloramphenicol	NEN
CIP	Boehringer MA
Deep Vent	Biolabs
dNTP	Pharmacia
DNase-free RNase	Boehringer MA
fluorescein conjugated avidin	Vectorlabs
fluoresein conjugated sheep anti-digoxoginin antibody	Boehringer MA
Glycogen	Boehringer MA
Klenow fragment	Boehringer MA
Polynucleotide kinase	Boehringer MA
Restriction enzymes were supplied by Gibco/BRL, AGS NE Biolabs and Boehringer MA.	

RNase A	Sigma
³⁵ S labelled dATP	NEN
T4 DNA ligase	Boehringer MA
T4 DNA Polymerase	Biolabs
Taq Extender	Boehringer, MA

• **Kits**

Chameleon [®] Site directed mutagenesis kit	Stratagene
GeneClean [®] kit	Bio 101
Maxi prep kit tip-500	Qiagen
Random Primed Labelling kit	Boehringer MA
RNA-Clean	AGS
T7 sequencing kit	Pharmacia

• **Plastic Material**

Corning bottles (125ml)	Corning
Electroporation cuvette (1,2 & 4 mm)	Eurogentec
Eppendorf tube (0.5ml, 1.5ml & Safe-lock)	Eppendorf
Falcon tube (15 & 50ml)	Falcon
Plastic tube (5 & 12 ml)	Greiner
"Quadriperm" tissue culture plates	Hereaus
Roller bottle (2L)	Falcon
Tissue culture bottle (150 & 250 cm ²)	Falcon
Ultracentrifuge tubes (5ml)	Beckman

• **Miscellaneous**

DEAE cellulose paper	Schleicher & Schuell
Electroporation apparatus Gene Pulser	Bio-rad
Films for sequencing/CAT assays and Northern	Kodak
"Genescreen" membrane	Dupont
"Superfrost" microscope slides	Menzel
Whatman filter paper	Whatman

- **Commonly used Buffers**

TAE (40 mM Tris-acetate, 1 mM EDTA)

TBE (90 mM Tris-borate, 2 mM EDTA)

TBS (25 mM Tris pH 7.4, 137 mM NaCl, 5mM KCl, 0.7 mM $\text{CaCl}_2 \cdot (\text{H}_2\text{O})_2$, 0.5 mM $\text{MgCl}_2 \cdot (\text{H}_2\text{O})_6$,
0.6 mM Na_2HPO_4)

HEPES (1 mM)

- **Special Equipment**

Centrifuge (eppendorfs)

Heraeus

Centrifuge(15ml & 50ml Falcons)

Hetisch, Heraeus

Centrifuge (125ml Cornings)

Sorval

Centrifuge and rotor (400ml bottles)

Sorval RC-4 plus GS-3 rotor

Ultra Centrifuge, rotor (5ml tubes)

Beckman, VTI 650 rotor

Speedvac vacuum drier

Bachofer

Fluorescence Microscope (confocal) & 60x lens

Zeiss

- **Cell Lines**

Jurkat Human T Cell Line

CD4⁺ Human T cell

Gillis and Watson 1980

Simian COS 7 Cell Line

Simian Kidney cell

Gluzman 1981

Hela Cell Line

Human Cervical Carcinoma

Gey *et al.* 1952

• Oligonucleotides

No./Name: 11/ B Q+F

5' AGC TGG AAG ATC TTC TAC CAT CGA
TGG CCG A

Description: Was annealed to oligo #12 and used to insert a Hind III, Bgl II, Cla I multiple cloning site into the existing Hind III at the 3' end of the MLV psi⁺ sequence. The Hind III site is destroyed at one end.

No./Name: 12/ BCH-R

5' AGC TTG GGC CAT CGA TGG TAG AAG
GAT CTT CC

Description: Was annealed to oligo #11 and used to insert a Hind III, Bgl II, Cla I multiple cloning site into the existing Hind III at the 3' end of the MLV psi⁺ sequence. The Hind III site is destroyed at one end.

No./Name: 19/ Psi-R

5' ATC CCG GAC GAG CCG CCA
TTC CTC TCC

Description: Anneals to the MLV psi sequence starting at nt. 164 and extending in the antisense orientation. Was used to sequence "backwards" into the 5'LTR to check for deletions of the U5 CRS sequence.

No./Name: 20/ Psi oligo

5' GAC TCA GCG GCC TCT T
TTC CTC TCC

Description: Anneals to the HTLV-I LTR starting at bp 533 of proviral DNA extending in the sense orientation. Was used for sequencing of LTR U5 regions to check for deletions of the CRS sequence.

No./Name: 29/ Int2

5' ACG AGC CCG CAA CTG TGT ACT AAA
TTT CTC TCC

Description: Anneals to the 3' end of the LTR extending in the antisense orientation. It was used to re-introduce the correct 3' IR while amplifying the 755b p 5' LTR (with oligo #91).

No./Name: 91/ Int1-1

5' ACC GCT GCA GTG CAG GTC GAC TCT
AGA GGA TCC CCG

Description: Anneals to pUC18 at the Xho I site. was used with #29 to PCR the 755b p 5' LTR out of plasmid No. 164.

No./Name: 92/ Int3-1

5' TAG TAC ACA GTT GGG GGC TCG TCC
GG

Description: Anneals to the 3' end of the LTR. It was used to re-introduce the correct 3' IR while amplifying the MLV psi⁺ (with oligo #11).

No./Name: 93/ Int1-2

5' ACC GCT CGA GAA GAG GCA GAT GAC
AAT GAC CAT GAG CC

Description: Anneals to Bluescript at the Xho I site. was used with #29 to PCR the 755b p 5' LTR out of Bluescript based plasmids. Similar to #91. Tm= 65°C.

No./Name: 94/ M13

5' GTA AAA CGA CCG CCA G
TTC CTC TCC

Description: Anneals to the M13 promoter region. Was used for sequencing inserts in the Bluescript plasmid.

No./Name: 95/ Psi300

Sequence unknown

Description: Anneals to MLV psi⁺ sequence approximately 300 bp from the 5' end, extending in the sense orientation. Was used for sequencing.

No./Name: 97/ CAT.A.seq5

5'

GCT	CGG	TAC	CC				

Description: Anneals to pBL-CAT2 just upstream of the CAT gene extending in the sense orientation. Was used to sequence the CAT-poly(A) insert.

No./Name: 98/ CAT.A.seq3

5'

GTT	GCA	TGC	CTG	C			

Description: Anneals to pBL-CAT2 just downstream of the poly(A) site, extending in the antisense orientation. Was used to sequence the CAT-poly(A) insert.

No./Name: 99/ T3

5'

AAT	TAA	COC	TCA	CTA	AAG	GG	

Description: Anneals to T3 promoter sequence. Was used for sequencing of inserts in Bluescript and together with the M13 oligo (#94) or T7 oligo (#149) for quick PCR screening of miniprep. bacterial colonies.

No./Name: 101/ CAT-1

5'

COC	AAG	CTT	GGA	GCT	TGG	CGA	GAT
TTT	CAG	G					

Description: Anneals to pBL-CAT2 just upstream of the CAT gene, extending in the sense orientation, introducing a Hind III site. Was used to PCR amplify the CAT gene without the poly(A). Used with oligo #102.

No./Name: 102/ CAT-2

5'

GAA	GAT	CTA	CCA	GGC	GTT	TAA	GGG
CAC	C						

Description: Anneals to pBL-CAT2 just upstream of the poly(A) site, extending in the antisense orientation, introducing a Bgl II site. Was used to PCR amplify the CAT gene without the poly(A). Used with oligo #101.

No./Name: 105/ RRE2

5'

GGT	CTA	GAG	TTT	ATT	GAG	CCA	TAT
GCG							

Description: Anneals to the HTLV-I LTR, to cover the poly(A) signal, extending in the antisense orientation. The oligo introduces an Xba I site at the 3' end of the U3 region. It was used together with oligo #93 to PCR amplify the U3 region. Tm= 50.5°C.

No./Name: 106/ RRE3

5'

CCT	CTA	GAT	CAA	CTC	TAC	GTC	TTT
GTT	TC						

Description: Anneals to the HTLV-I LTR, to cover the poly(A) site, extending in the sense orientation. The oligo introduces an Xba I site at the 5' end of the U5 region. It was used together with oligo #107 to PCR amplify the U5 region. Tm= 53°C.

No./Name: 107/ RRE4

5'

CCT	COS	AGC	TCA	CTG	TGT	ACT	AAA
TTT	CTC	TOC					

Description: Anneals to the HTLV-I LTR at the 3' end of the U5 region, extending in the antisense orientation. The oligo introduces a Sac I site at the 3' end of the U5 region. It was used together with oligo #106 to PCR amplify the U5 region. Tm= 52.7°C.

No./Name: 108/ KpnI

5'

CTA	TAG	GGC	GAA	TTG	GGT	GCG	GGG
COC	COC	CTC	GAG				

Description: Anneals to the Bluescript plasmid covering and destroying the Kpn I site, extending in the sense orientation to include the Hind III site. Was used as the "selection" primer during SDM with oligo #109. The oligo is non-phosphorylated.

No./Name: 109/ Hind III

5'

GCG	TGG	TCC	GGG	ATA	AGC	TTA	COC
CTG	COC	AGG	GAC	C			

Description: Anneals where the 5' LTR borders the MLV psi+ sequence, extending in the sense direction to insert a Hind III site just downstream of the 5' LTR. Used in an SDM protocol with oligo #108. The oligo is non-phosphorylated.

No./Name: 113/ U3-Mun

5'

COC	CCA	ATT	GGG	ATC	CAG	CAG	GAG
TCT	ATA	AAA	GCG				

Description: Anneals to the HTLV-I LTR at the 5' end of the R region, extending in the sense orientation to exclude the poly(A) signal but introduce Mun I and BamHI sites. Used together with oligo #114.

No./Name: 114/ U5-Mun

5'

COC	CAA	TTG	AGA	TCT	AGC	AGG	GTC
AGG	GTC	AGG	CAA	AGC	G		

Description: Anneals to the HTLV-I LTR at the 3' end of the R region, extending in the antisense orientation to exclude the poly(A) site but introduce Mun I and Bgl III sites. Used together with oligo #113 to PCR the RRE as a Mun I fragment.

No./Name: 115/ 5'CAT-rev

 5'

G	C	T	C	C	T	G	A	A	T	C	T	C	G	C		

Description: Anneals just upstream of the CAT gene, extending in the antisense orientation. This oligo is complementary to #101 which was used to PCR the CAT gene from pBL-CAT2. Was used to sequence MLV Psi fragments once inserted into the SDMed Hind III site.

No./Name: 116/ ORI-XhoI

 5'

C	C	G	C	T	C	G	A	G	C	A	T	C	T	C	A	T	G	C	T	G	A	C
T	A	A	T																			

Description: Anneals to the 5' end of the SV40 origin of replication, extending in the sense orientation to introduce an Xho I site. Used together with oligo #117. Tm= 50.5°C.

No./Name: 117/ ORI-SalI

 5'

A	C	G	C	G	T	C	G	A	C	A	A	G	C	C	T	C	C	A	A	T	C	T	A	C	T	A
C	T	T	C																							

Description: Anneals to the 3' end of the SV40 origin of replication, extending in the antisense orientation to introduce a Sal I site. Used together with oligo #116.

No./Name: 119/ pBS.reverse

 5'

G	G	A	A	A	C	A	G	C	T	A	T	G	A	C	A	T	G									

Description: Anneals to the Bluescript plasmid covering the β -galactosidase ATG site at bp 816. Used to sequence inserts in the Bluescript plasmid.

No./Name: 146/ R-SD

 5'

C	C	C	A	A	G	C	T	T	G	G	T	T	G	A	G	T	C	G	C	G	T	T	C			
T	G																									

Description: Anneals to the HTLV-I LTR just upstream of the Splice donor site. Used with oligo #115 to detect splicing events in constructs containing the Psi312 fragment.

No./Name: 147/ R-SD.2

 5'

C	C	C	A	A	G	C	T	T	G	G	T	T	G	A	G	T	C	G	C	G	T	T	C			
C	T	G	C																							

Description: Same as oligo #146 but with an extra 3 "C" nucleotides to increase the Tm (61°C).

No./Name: 148/ -

 5'

G	C	T	T	C	T	T	A	G	C	T	C	T	G	A	A	T	C	T	C							
G	C	C																								

Description: Same as oligo #115 but with an extra 9 nucleotides at 5' end to increase the Tm (64°C).

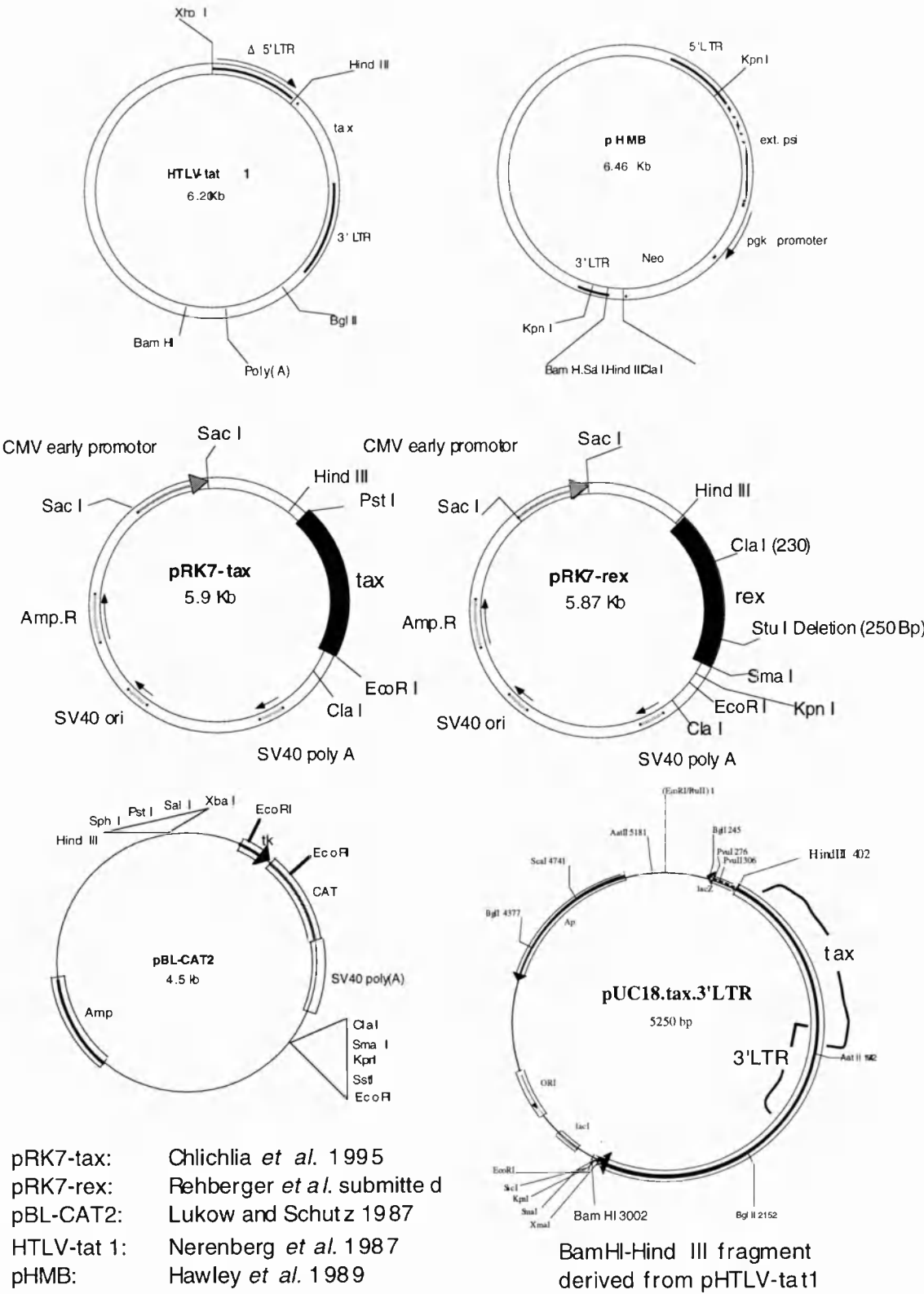
No./Name: 149/ T7

 5'

G	T	A	A	T	A	C	G	A	C	T	C	A	C	T	A	A	G	G	G	C						

Description: Anneals to T7 promoter sequence. Was used for sequencing of inserts in Bluescript and together with the T3 primer (#99) for quick PCR screening of mini prep. bacterial colonies.

• Plasmids used



• Growth of Bacterial Cultures

Liquid bacterial cultures were grown at 37°C, with 200-250 rpm shaking. LB medium was prepared according to Maniatis *et al.* Ampicillin was used at a concentration of 0.1mg/ml (100mg/ml stocks were stored at -20°C). Depending on the size of culture, various culture vessels were used:

Purpose	Vessel	Volume
Post-electroporation incubation	12 ml plastic tube	1 ml
Mini prep	12 ml plastic tube	2 ml
Midi prep	250 ml Conical flask	50 ml
Competent cells	2 L Conical flask	400 ml
Maxi prep	2 L Conical flask	100-500 ml

Maxi prep culture volumes varied depending on the plasmid type being grown. Smaller volumes were used when growing up plasmids with a high copy number.

To plate out transformed bacteria, LBamp agar plates were prepared. A 1.2% solution of agar in 1x LB medium was autoclaved (120°C, 20 minutes) and allowed to cool to 50°C. Ampicillin was added to a final concentration of 0.1mg/ml. 20 ml of LBamp agar was then poured into each sterile 10 cm petri dish. Plates were stored for up to 2 months at 4°C.

• Preparation of Electro-competent Bacteria

Competent cells (*E. Coli* strain HB101) were prepared from a 400 ml LB culture which was grown to an optical density (O.D) at 600 nm, of 0.3 - 0.5 units. Bacteria were maintained in an exponential growth phase, having first been plated out the evening before on an LB agar plate. Early the next morning, 2 ml of LB medium was inoculated with a single colony and grown for approximately 2-3 hours, whereon 500 µl was used to inoculate a 400 ml LB culture. A further 3-4 hour incubation was then required before the optimal OD₆₀₀ was reached.

All solutions, pipettes, vessels and rotors were pre-chilled to 4°C. Centrifugation steps were all carried out at 4°C. The culture was first chilled on ice for 10 mins before centrifugation at 4000 rpm for 15 mins in a Sorvall GS-3 rotor. The supernatant was removed and the cells resuspended in 10 ml of 1mM HEPES (pH 7) solution. The volume was then increased to 300 ml with HEPES and re-centrifuged at 5000 rpm in the same rotor. This washing step was repeated once. Following this third centrifugation step, the supernatant was removed and the cells resuspended in 30 ml of sterile 10% glycerol and transferred to a 50 ml Falcon tube. This was then centrifuged at 3000 rpm for 15 mins in a

Heraeus "Variofuge" (max. of 6000 rpm). The supernatant was removed and the cells resuspended in 800 μ l of 10% glycerol. The cells were aliquoted, immediately snap-frozen in liquid nitrogen and transferred to -70°C , where they were kept for up to 2 months. The transformation efficiency of each fresh batch was tested by electroporating 0.1ng of pUC 18 plasmid DNA. An efficiency of 1 million colonies per μ g DNA was obtainable.

This HEPES washing procedure removes the salt (from the LB medium) which, if present during the electroporation procedure, would lead to a reduced electroporation efficiency.

• Electroporation of Competent Cells with Plasmid DNA

Electroporation was carried out using 40 μ l of electro-competent cells and 1-3 μ l of plasmid DNA-containing solution. If the DNA was derived from a previous plasmid prep then 10-100 ng was used. If, however, the DNA was derived from a ligation reaction, then 1-2 μ l were used. The ligation mix contains salt, thus the volume of mix should be kept below 2 μ l. Alternatively, the ligation mix can be phenol-chloroform extracted, ethanol precipitated and resuspended in a small volume of distilled water, before electroporation. Sterile electroporation cuvettes with a 0.1 or 0.2 cm electrode gap were pre-chilled on ice. The competent cells and DNA were then mixed by gentle pipetting in an eppendorf tube and transferred to the cuvette. Using the Bio-rad Gene Pulser device, the mix was pulsed at 2.5kV with a capacitance of 960 μ F yielding an optimal time constant of 4.4 - 4.6 mS. This is the time taken for the pulse to cross the electrode gap. High salt concentrations promote a quicker conductance which leads to a lower transduction efficiency.

Immediately following pulsing, 1ml of sterile ice cold SOC medium (bactotryptone 20g/L, yeast extract 5g/L, NaCl 0.1 M, KCl 2.5 mM, MgCl 10 mM, MgSO₄ 5 mM) was added, the cells gently mixed and transferred to a 12 ml plastic tube for 60 minutes at 37°C with shaking. After this time, 100 μ l was plated out on LB_{amp} agar plates, the remainder was centrifuged (4000 rpm in an Eppendorf mini centrifuge) for 2 minutes. 800 μ l of the supernatant was removed, the cells being resuspended in the remaining 100 μ l. These were then also plated out on LB_{amp} agar plates. Both plates were inverted and incubated at 37°C overnight (16 hours) to allow ampicillin resistant colonies to grow.

- **Plating out bacteria on LB (LB-amp) Plates**

Bacteria from frozen stocks were plated out via "streaking". Here an inoculating stick was used to transfer the concentrated bacteria to the plate as 3 parallel lines at a tangent to the plates circumference. A fresh inoculating stick was then taken to make new streaks that started by crossing the old streaks. This was continued until the plate had been completely rotated to give 5 to 6 sets of tangents ensuring that at some point in the dilution procedure, single colonies would appear. The plates were incubated for 16 hours at 37°C to allow colonies to grow.

Bacteria from electroporations were centrifuged gently at 4000 rpm (eppendorf centrifuge) in 1.5 ml eppendorf tubes. The supernatant was carefully removed so as to leave approx. 100 µl of supernatant behind (SOC medium). The bacteria were then carefully resuspended in this medium via pipetting before being transferred to the surface of the LB-amp plate. This 100 µl was then spread across the surface of the plate using a sterile inoculating loop. Plates were allowed to air-dry before being incubated for 16 hours at 37°C.

- **Small Scale ("TELT") Plasmid DNA Preparation**

Following electroporation and overnight selection on LB_{amp} plates colonies were picked and used to inoculate both a fresh LB_{amp} plate and a 2 ml volume of LB_{amp} medium in a 12 ml plastic tube. The plate was incubated at 37°C overnight and the 12 ml tube at 37°C with shaking for a minimum of 5 hours. A quick (same day) mini prep procedure was used which yielded a small but sufficient amount of plasmid DNA to allow 5-10 restriction enzyme digestions or 2-3 sequencing reactions. The advantage of this method being that batches of 24 preps can be easily prepared in under 2 hours on the day of inoculation allowing subsequent restriction digest analysis and inoculation of 400 ml LB for a large scale plasmid prep on the same day, effectively saving one working day.

First, the mini cultures were spun down in eppendorf tubes (8000 rpm for 1 min) and the supernatants removed via aspiration. 100 µl of TELT buffer (2.5 M LiCl, 50 mM Tris pH 8, 4 % (v/v) Triton X-100, 62.5 mM Na₂ EDTA) was added to each tube using an eppendorf multi-pipette. The tubes were transferred to an eppendorf shaker and shaken for 30 seconds to resuspend the bacteria. In this buffer the bacteria are also lysed. Immediately, 100 µl of phenol-chloroform (1:1) was added and the tubes shaken once more - this time for 1 minute to ensure good mixing of the organic phase with the lysate proteins. This was then centrifuged at 13000 rpm for 5 minutes at RT to separate the phases. The upper phase was transferred to a fresh eppendorf tube and precipitated with 300 µl of 100% ethanol. This was then immediately centrifuged at 13000 rpm for 3 mins. The supernatant

was removed and the pellet washed with 500 μ l of 75% ethanol to remove the large amounts of salt (LiCl) present. A second wash step was optional. Following the final centrifugation, the supernatant was removed, the pellet allowed to dry briefly in a "Speedvac" drier before being resuspended in 30-50 μ l of TE (pH7.4). For restriction enzyme digestions 5-10 μ l was used with 1 μ l of DNase-free RNase in addition to the suitable restriction enzyme.

• Restriction Enzyme (RE) Digestions

Most RE manufacturers supply enzymes with an activity of 10 or 20 units/ μ l in glycerol (for stability). One unit is required to cleave one μ g DNA in one hour at the optimum (usually but not always 37°C) temperature. Digestions were carried out with an excess of 3-5 fold. This excess activity can either be achieved by extending the incubation period to 3 or 5 hours instead of one or by using 3-5 times more enzyme in a one hour digestion. In practice, shorter digestions were preferred for mini prep DNA. Glycerol concentrations over 10% seriously decrease the activity of restriction enzymes. For this reason, the volume of enzyme was kept below 10 % of the total reaction volume. (NB. The RNase used in digestions of mini prep DNA is also stored in glycerol.)

• Large Scale (Midi) Plasmid DNA Preparation

When a large amount of DNA was required, which did not need to be pure enough for the transfection of eukaryotic cells, a "midi prep" was carried out. 50ml of LB was inoculated with a single colony from an LB_{amp} plate. This was grown up overnight at 37°C with shaking. The following day, the culture was transferred to a 50ml Falcon tube and centrifuged for 10 minutes at 4000 rpm in a Heraeus centrifuge. The supernatant was removed and the bacteria resuspended in 9 ml of chilled GTE buffer (50 mM glucose, 25 mM Tris pH 7.6, 10 mM EDTA, 400 μ g/ml RNase A). Next, 9 ml of lysis buffer (200mM NaOH, 1% SDS) was added, mixed carefully and kept at RT for 10 minutes. 9 ml of Neutralizing buffer (5M Acetic acid, 3M KAc) was then added and again carefully mixed. This was incubated on ice for 20 minutes and then centrifuged for 30 minutes at 17000 rpm in an SS34 rotor in a Sorvall refrigerated centrifuge at 4°C. The supernatant was removed and precipitated with 0.7 volumes of Isopropanol on ice for 30 minutes. This was then centrifuged at 5000 rpm for 30 minutes at 4°C in 50 ml Falcon tubes to pellet the nucleic acids. The supernatant was removed and the inside wall of the tube dried with a sterile paper tissue. The pellet was resuspended in 2 ml of TE buffer (10 mM Tris pH 7.4, 1 mM EDTA). 2 ml of 5M LiCl was then added and mixed well. Following a 30 minute

incubation on ice, this was centrifuged for 30 minutes at 5000 rpm at 4°C to pellet the precipitated RNA. The supernatant (4 ml) was then transferred to a fresh 15 ml Falcon tube and precipitated with 2 volumes of 100% ethanol on ice for 30 minutes. This was centrifuged for 30 minutes at 4500 rpm (Heraeus) at 4°C to pellet the DNA. The supernatant was discarded and the DNA pellet resuspended in 500 µl TE. This was then sequentially extracted with one volume of phenol:chloroform (1:1) and then with one volume of chloroform:Isoamylalcohol (24:1). The final upper phase was then precipitated with 2 volumes of 100% ethanol at -20°C. This was then centrifuged at 4500 rpm at 4°C. The supernatant was removed, the pellet washed twice with 2 ml of 75% ethanol and re-centrifuged. The supernatant was removed, the pellet dried briefly in a "Speedvac" vacuum drier and resuspended in 500 µl of TE (pH 7.4). The concentration of DNA was then determined spectrophotometrically at 260 nm. An OD₂₆₀ of 1.0 is equivalent to a concentration of 50 µg double stranded DNA / ml. Total yields of 1-2 mg were obtainable. Midi prep solutions were almost completely free of RNA, containing only a low level of protein but because of the presence of highly toxic bacterial lipopolysaccharides (LPS), they were not suitable for eukaryotic transfections. They were, however, ideal as substrates for *in-vitro* sub-cloning procedures.

• Large Scale (Maxi) Plasmid Preparation

To prepare larger amounts of plasmid DNA for use in eukaryotic transfections, two protocols were available. The traditional method, which can yield large amounts (1-2 mg) of plasmid DNA is a two-day process. The first day is similar to the method described above. At the end of the first day, the DNA is centrifuged in a CsCl gradient to separate the DNA from RNA and protein. The DNA bands can be removed from the polyalomer tubes using a syringe and needle and then further processed to remove the ethidium bromide (used to visualize the DNA bands) and high concentration of CsCl salt. To obtain ultra pure plasmid DNA a second CsCl centrifugation should be performed.

An alternative to this method, which also yields pyrogen-free plasmid DNA, albeit in lower amounts (500-1000 µg), is the affinity chromatography-based method introduced by Qiagen Ltd. This process takes 2-3 hours and produces ultra clean DNA which was used to gain very reproducible results in eukaryotic transfection protocols. The level of purity is claimed to be equivalent to two rounds of CsCl purification and is sufficient for use in *in-vivo* human gene therapy protocols.

For the Qiagen method, it is important not to use excessive amounts of bacteria. As the columns used only bind 500 or 1000µg plasmid DNA, it is recommended to use significantly smaller culture volumes when growing up plasmids with a high copy number.

With such plasmids, 500 µg is easily obtained from a 50 ml culture. Use of 400-500ml is only recommended for low copy number plasmids such as pBR322.

Bacteria were harvested by centrifugation and resuspended in 10 ml of chilled buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). 10 ml buffer P2 (0.2M NaOH, 1% SDS) was then added to lyse the bacteria for 5 mins at RT. 10 ml of chilled neutralization buffer P3 (3M Potassium Acetate, pH 5.5) was then added, mixed gently and centrifuged at 17000 rpm for 40 mins at 4°C in an SS34 rotor in a Sorvall centrifuge. A "TIP-500" column was then equilibrated with 10 ml of equilibration buffer (750 mM NaCl, 50 mM MOPS, 15% ethanol pH 7.0, 15% Triton X-100). The supernatant was applied to the column, the DNA binding to the DEAE-based substrate. Three wash steps (each 10ml of 1M NaCl, 50 mM MOPS, 15% ethanol pH 7.0) were carried out to remove unbound protein, RNA etc. The remaining DNA was then eluted from the column with 15 ml of elution buffer (1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol pH 8.5) and precipitated with 0.7 volumes of isopropanol. This was centrifuged at 5000 for 30 mins at 4°C (Heraeus) to pellet the DNA. The supernatant was then removed and the pellet washed twice with 75% ethanol. The pellet was finally dried briefly and resuspended in 300-500 µl TE (10 mM Tris-HCl, 1 mM EDTA pH 8). This solution was then assayed at OD₂₆₀ for both DNA concentration and purity.

• Freezing Bacterial Stocks

Plasmid-containing bacteria were harvested prior to maxi plasmid DNA preps, pelleted at 3000 rpm for 5 minutes at room temperature and resuspended in 200 µl of LB medium. 600 µl of 86% glycerol was then added and mixed. These aliquots were then stored at -70 °C for future maxi prep inoculations.

• Gel Electrophoresis of Plasmid DNA

Plasmid DNA fragments were separated via gel electrophoresis through either 0.8% or 1.2% agarose gels. Agarose was heated in 1xTAE buffer in a microwave oven until no undissolved agarose remained. Once the agarose had cooled to approximately 50°C, ethidium bromide was added to a final concentration of 0.5 µg/ml. This solution was then kept at 50°C (for up to a week) until needed. Agarose gel loading buffer (0.2% SDS, 10 mM EDTA, bromophenol blue 0.004%, glycerol 10% pH 7.5 final concentration) was added to each sample to facilitate loading and also to allow the visualization of the running front. Gels were electrophoresed at 100V /90 mA in a Pharmacia mini gel apparatus.

- **Polyacrylamide Gel Electrophoresis (PAGE)**

Polyacrylamide gels were used to run the products of DNA sequencing reactions. An 8% gel was made by taking 8 ml of a 40% acrylamide / 2% BIS stock solution. Ion-exchange beads were kept in the stock solution to help prevent polymerization. To this was added 19.4 g ultrapure urea, ddH₂O up to 40 ml and some Serdolit ion-exchange beads. This was heated at 50°C for 15 mins with an electronic stirring bead and then filtered through Whatman filter paper onto 8 ml of 5 times TBE buffer. While this solution was on ice, 160 µl of 10% ammonium persulphate (APS) and 16 µl of TEMED was added. This was mixed and then de-gased by quickly drawing the solution into a 50 ml syringe through a hyperdermic needle. The solution was then injected slowly between the two glass gel plates and allowed to polymerize at room temperature for approximately 60 minutes.

- **Determination of DNA and RNA Concentrations**

5 µl of the sample nucleic acid solution was brought to 700 µl with distilled water. The photometric absorbances at 260 and 280 nm were then measured relative to a distilled water blank. The concentration (µg/ml) of dsDNA is calculated by multiplying the A₂₆₀ by the dilution factor and by the standard absorbtion coefficient for DNA (50). For RNA and single-stranded (ss) DNA the absorbtion coefficient is 37.

i.e. A₂₆₀ of 1.0 results from a dsDNA solution of concentration 50 µg/ml

A₂₆₀ of 1.0 results from a ssDNA /RNA solution of concentration 37 µg/ml

A measure of the purity of the DNA or RNA can be made by dividing the A₂₆₀ by A₂₈₀.

Values of 1.7 - 1.8 for DNA and 2.0 - 2.1 for RNA indicate good purities. Lower ratios (1.5-1.6) indicate the presence of protein.

DNA fragment concentrations were estimated by running various dilutions of the fragment against a known amount of digested lambda marker DNA and comparing band intensities.

- **Purification of DNA Fragments**

Following digestion of plasmid DNA by restriction enzymes (RE), the DNA fragments could be isolated and purified. The method of purification depended upon the size of the fragment and whether RE digestion led to one or more fragments as well as the difference in size between the fragments.

Direct Genecloning:

Where a plasmid had been digested in preparation for insertion of another DNA fragment (insert), either by linearization or by cutting twice to release a small piece of the plasmid (multiple cloning site), it was not necessary to run the fragment(s) on an agarose gel. The

DNA were purified directly from the RE digestion mix using the "GeneClean" kit. 3 volumes of sodium iodide solution was added to the digestion mix. 5 μ l of "glassmilk" (silica substrate) was added to this mixture and incubated at room temperature for 5 minutes. Glassmilk binds DNA fragments larger than 500 bp with efficiencies of up to 90%. Small fragments effectively do not bind. The glassmilk was pelleted by centrifugation and washed 3 times with 600 μ l of -20°C "New Wash" solution - 70% ethanol with high salt concentration. This removed the sodium iodide salt but ensured that the DNA remained bound to the glassmilk. The DNA was eluted by resuspending the glassmilk in low salt solution (distilled water or TE buffer pH 7.4) and incubating at 50°C for 10 minutes. A small portion of the eluate was then run on an agarose mini gel to check the DNA yield.

Gel and GeneCcleaning:

Fragments larger than 500bp were purified by first running the mixture on an agarose gel and cutting out the band of interest. It is important to excise the band in the smallest amount of agarose possible. This was transferred to an eppendorf tube and weighed. Where the agarose weighed more than 300 mg then it was divided into two fractions and processed separately. 3 volumes of sodium iodide was added to the agarose and heated at 50°C until the gel just melted. Glassmilk was then added and further processed as described above.

Gel and Electrolution:

Small DNA fragments (20-2000 bp) were removed from the gel by cutting a slit in the gel, just in front of the band, inserting a small piece of DEAE cellulose paper and running the gel further so that the band ran "on to" the paper. The paper was then washed well in distilled water to remove any remaining agarose gel, placed at the bottom of an eppendorf tube and just covered with a solution of 1M NaCl and heated to 65°C for 30 mins to elute the bound DNA. DNA fragments larger than 2.5 kb do not elute very well from the paper as they are too tightly bound. The eluate was extracted with one volume of phenol:chloroform (1:1) and precipitated with 0.2 volumes of 10M ammonium acetate and 2.5 volumes of 100% ethanol. This was then centrifuged at 13000 rpm for 15 minutes to pellet the DNA which was then washed twice with 500 μ l of 70% ethanol, briefly dried under vacuum in a Speedvac and resuspended in either distilled water or TE buffer, pH 7.4.

- **"Blunting" of Restriction endonuclease digested DNA ends**

Digestion of DNA with a restriction endonuclease typically results in production of either a 5' or 3' overhang. This "sticky" end can be "blunted" in a reaction catalysed by T4 DNA polymerase. Normally, the sticky end is a 5' overhang. In this case the polymerase will extend the 3' end until flush - or blunt. In the case of a 3' overhang, the enzyme will degrade it back until blunt. This is possible because the enzyme has both a 5'→3' polymerase activity and 5'↔3' single strand exonuclease activities. In the presence of dNTPs, the enzyme's polymerase function dominates.

3-4 µg of digested DNA in a total volume of 39 µl was added to an eppendorf tube. To this was added 5 µl of dNTPs (6 mM each), 5 µl of 10x Klenow buffer and 1 µl of T4 DNA polymerase (5 units). This was incubated at 15°C for 20 minutes after which the reaction was stopped upon addition of 1 µl of 0.5M EDTA. 0.1 volumes of 3M sodium acetate (pH 4.8) was added and the mixture extracted first with one volume of phenol:chloroform (1:1) and then with one volume of chloroform: isoamylalcohol (24:1). The final aqueous phase was then precipitated by addition of 0.7 volumes isopropanol and incubation for 10 minutes at room temperature. Following centrifugation, the pellet was washed in 100 µl of 70% ethanol, briefly dried under vacuum and resuspended in 10-20 µl of TE (pH 7.4). 1 µl was then run on an agarose gel to estimate the new DNA concentration.

- **Dephosphorylation of Plasmid DNA ends**

When ligating a DNA fragment (insert) into a single site of a plasmid (vector), one can either use a 5:1 insert to vector ratio and be prepared to do 24 mini preps to determine which bacterial colonies contain the desired vector-insert combination or modify the ends of the vector DNA so that they are no longer able to re-circularize (and give rise to ampicillin resistant colonies). The ends were modified by a phosphatase enzyme (purified from calf intestine) known as CIP. This removes the Phosphate group from each end of the vector DNA making re-circularization impossible. The insert DNA, however, still has both phosphate and hydroxyl groups at each of its ends and is thus able to ligate with the vector DNA. In this case a 1:1 vector to insert ratio is sufficient and should result in all ampicillin resistant colonies containing the vector and one or more inserts in either of the possible orientations. It should be noted that phosphatasing leads to a marked reduction in ligation efficiency.

2-3 µg of vector DNA in a total volume of 17 µl was added to an eppendorf tube. To this were added 2 µl of 10x CIP buffer and 1 µl of CIP enzyme (2-3 units). This was incubated for 30 minutes at 37°C and the reaction stopped upon addition of 2 µl of EGTA (100 mM

stock concentration) and incubation for 15 minutes at 75°C, to inactivate the CIP. 1 µl of glycogen carrier and 0.2 volumes of 10M ammonium acetate were added. The mixture was then extracted, first with 1 volume of phenol:chloroform (1:1) and then with one volume of chloroform:isoamylalcohol (24:1). The final aqueous phase was precipitated with 2.5 volumes of 100% ethanol, washed twice with 70% ethanol, dried briefly under vacuum and resuspended in 10 µl of TE (pH 7.4). 1 µl was then run on an agarose gel to estimate the new DNA concentration.

• Kinasing of DNA ends

DNA fragments destined for ligation into a plasmid are usually digested with one or two restriction enzymes to yield either sticky or blunt ends. These ends have terminal phosphate groups, essential for the subsequent ligation reaction. In some cases the inserts are generated via the PCR, and if the PCR polymerase used has a proof-reading function (such as Vent or pfu) then the PCR product ends will be blunt. If restriction enzyme cleavage sites were not included at the 5' ends of both oligos, then the fragment must be ligated with one or two blunt ends. A PCR generated blunt end lacks the terminal phosphate group and can therefore be modified prior to ligation.

100 pmoles of DNA fragment in 12µl dH₂O was added to an eppendorf tube. To this was added 2 µl of 10x buffer (0.2 M Tris pH 7.6, 0.1 M MgCl₂, 10 mM DTT), 2 µl of 160 µM ATP and 1µl of polynucleotide kinase. This was incubated for 30 minutes at 37°C, and the reaction stopped upon addition of 2 µl of 0.5 M EDTA.

• Ligation of DNA Fragments

The exact protocol used for a ligation reaction depended upon the relative lengths of the fragments, the nature of the ends (sticky, RE blunt, T4 pol blunt, PCR blunt and/or phosphatased) and the abundance of each fragment. The optimal molecular ratio of vector to insert could only be estimated once small portions of each fragment had been run next to one another on an agarose mini gel.

Reaction One: Vector and insert each have two different sticky ends (eg. Bam HI & EcoRI). In this case, a 1:3 molecular ratio was sufficient in a standard reaction at 16°C overnight. The standard reaction consisted of 200 - 400 ng DNA, T4 DNA ligase buffer (50 mM Tris-HCl pH 7.6, 10 mM MgCl, 10 mM DTT, 50 µg/ml BSA), 5 mM ATP, dH₂O up to 9 µl and 1 µl T4 DNA ligase. Before addition of the ligase, the mix was incubated at 40-50°C for 5 minutes and then placed directly on ice for 5 minutes. This melted any cohesive ends which may have formed between vector and vector as well as between insert and insert and thus increased the efficiency of correct (vector-insert) ligations. For

each ligation reaction two control reactions were included - one lacking insert DNA and the other lacking the ligase enzyme. When the ligation reaction was complete, 2.5 µl of each of the three reactions was run on an agarose mini gel. From this it could be determined whether the vector and insert fragments had ligated. The reaction including vector, insert and ligase produced many bands, identification of the correct vector-insert band being difficult. The ligase minus lane shows the positions and quantities of both the vector and insert bands "before ligation". The insert minus lane shows the position of the vector-vector multimers which are also produced if the vector DNA is not CIPed. By subtracting the control bands from those of the target reaction, the correct vector-insert band can be identified more easily.

Reaction Two: Vector and Insert have one blunt and one sticky end (eg. EcoRV & EcoRI). This reaction is the same as that described above. The sticky ends ligate more readily than the blunt ones but it is still a directed cloning.

Reaction Three: Vector and insert ends are all the same or compatible (eg. Bam HI or Bam HI & Bgl II). In this case vector and insert ends can ligate with themselves as well as with one another. Furthermore, the insert is able to ligate in in both orientations and with multiple copies. The orientation of the insert can be determined by screening the colonies following electroporation. There are two ways of increasing the chances of correct, vector-insert ligates. The vector molecules can be treated to remove their terminal phosphate groups. This prevents vector-vector ligations but also leads to a reduced ligation efficiency. The efficiency of dephosphorylation can be analysed by looking at the insert minus control lane of the agarose mini gel after ligation. Alternatively, the vector : insert can be increased to 1:6, or with very small fragments up to 1 : 10.

Reaction Four: Vector and insert both have blunt ends. In this case, intra-molecular ligation is always kinetically favoured. Three measures can be taken to optimize the chances of inter-molecular ligations. Firstly, the reaction temperature can be dropped to 12°C and allowed to proceed for more than 24 hours. Secondly, a higher insert to vector ratio can be used (6:1 to 10:1). Thirdly, the effective concentration of the DNA ends can be artificially increased by adding polyethylene glycol (PEG 6000) to the reaction mixture to a final concentration of 5 %.

• Sequencing of Plasmid DNA

DNA was sequenced using the T7 sequencing kit from Pharmacia.

1-2 µg of DNA was denatured with NaOH (final concentration 200 mM). The addition of sodium acetate neutralized the pH and the mixture was then precipitated with 2 volumes of 100% ethanol. The pellet was resuspended in 10 µl TE and kept on ice. The primer

was annealed to the template at a molecular ratio of 5:1 using the kit's annealing buffer and incubation first at 37°C for 30 minutes and then at room temperature for 30 minutes. The stock T7 polymerase was diluted 1:4 using the kit's enzyme dilution buffer and kept on ice. Next the enzyme pre-mix was pipetted together in an eppendorf tube on ice. This consisted of diluted enzyme, ³⁵S labelled dATP and the kit's labelling mix. After a 5 minute incubation at room temperature, a portion of the mix was added to each of four wells of a 96 well plate. The wells contained a nucleotide mixture of three dNTPs and one ddNTP, each well containing a different ddNTP as the chain terminator. The short mix (up to 400 nt extension) was always used as this was found to give the most accurate and reproducible results. The reaction was incubated at 37°C for 5 minutes and then stopped upon addition of 5 µl of the kit's stop buffer, which later acted as loading buffer.

An 8 % polyacrylamide gel was prepared. The polymerized gel was then mounted upright in its electrophoresis chamber, which was filled with TBE buffer and allowed to "pre-run" for 30 minutes. To ensure an even gel temperature, an aluminium plate was clamped to the rear gel plate to facilitate heat transfer. Following loading and electrophoresis at 70 Watts/ 2.5 kV, the gel plates were separated and the gel transferred to a piece of Whatman blotting paper and dried under vacuum at 80°C for 60 minutes. Once dry, the blot was placed in an autoradiography film box and exposed over night at room temperature. The sequence was then read and entered into the DNA Strider computer program for further processing.

• Polymerase Chain Reactions (PCR)

During the PCR, at least two specific oligonucleotides (or primers) anneal to a DNA template and are elongated by a highly temperature stable DNA polymerase. As each oligonucleotide anneals to a different template strand and extends towards the other - through successive cycles of oligo annealing, polymerization and strand de-naturation - the resulting PCR product will be one or more double stranded DNA fragments that incorporate the two oligos at their 5' ends.

•Standard PCR: Template DNA, PCR primers (each 0.2 µM), dNTP (20 µM), 10x polymerase buffer, DNA polymerase (1µl), MgSO₄ (2mM) and dH₂O to a final volume of 50 to 100 µl. 2 drops of mineral oil are added to prevent evaporation of the aqueous (lower) phase. The cycling reactions are normally separated into three stages. Stage one: De-naturation at 95°C for 3-4 minutes, annealing at the optimum temperature for 2 minutes and extension for 3 minutes at 72°C. Stage two: 25-35 cycles of 1 minute at 95°C, 1 minute at annealing temperature and 1 minute extension at 72°C. Stage three consists simply of an additional extension incubation at 72°C for 4-6 minutes to ensure

that all PCR primers have been fully extended. The extension time correlates to the predicted length of the PCR product, while the annealing temperature is specific for the length and sequence of the two primers used. For specific annealing, a temperature 3 - 4° below the maximum should be chosen.

Depending on the PCR application, one of three different DNA polymerase enzymes were used. For very sensitive detection of normal sized templates (up to 2 Kb), a Taq polymerase was chosen. Where the PCR product was required for a subsequent sub-cloning step, then a polymerase with proof reading such as Deep Vent DNA polymerase was essential. For sensitive detection and mutation-free amplification, Taq Extender was used. This is an optimized mixture of the standard Taq polymerase and a polymerase with proof reading. Polymerases with proof reading produce blunt ended PCR products, whereas, Taq polymerase always adds a thymidine nucleotide, in a template independent manner to the 3' end. Taq extender thus yields PCR products with both blunt and overhanging ends.

•Quick screening of mini prep colonies: Following a subcloning reaction, positive colonies resulting from electroporation were quickly screened using the PCR. By selecting oligos that annealed within the insert and/or the plasmid, the appearance and size of the resulting PCR product indicate not only the presence or absence of the insert, but also the number of inserts as well as the insert's orientation (useful if all ends are compatible).

A cocktail containing both primers (each 1μM), 1x PCR buffer, dNTPs (20μM), DNA polymerase, MgSO₄ (4μM) and dH₂O was prepared and aliquoted (20 μl) into PCR tubes. Using a P20 Gilson pipette with a yellow tip, bacterial colonies were picked from the agar amp plate, transferred to a fresh, numbered agar amp plate and then used to pipette up and down the 20 μl PCR volume. This inoculation was sufficient to transfer enough plasmid DNA for PCR amplification. The standard cycle conditions were used, allowing 200 - 300 colonies to be rapidly screened. Maxi preps were then inoculated by picking the positive numbered colonies from the transfer agar amp plate.

• Site Directed Mutagenesis (SDM)

When it was necessary to introduce DNA sequence mutations into plasmids, and convenient restriction endonuclease cleavage sites were not available (making PCR difficult), the Chameleon[®] Site directed mutagenesis kit was used.

Two oligos were chosen. The first (mutation) oligo contained the mutation to be introduced. The second (selection) oligo covered and destroyed a unique restriction site within the plasmid. Unlike the PCR reaction oligos, the SDM oligos are 5' phosphorylated, annealed to the same template strand and were extended (by DNA polymerase I) in the

same direction. This resulted in two extended primers which - through being phosphorylated - were able to join up to one another to create a mutated strand. This was digested with the restriction endonuclease, whose site was destroyed by the selection oligo, purified and used to transfect *E. Coli* (strain XL1-blue). These cells, when transfected with an M13 ORI-containing double-stranded plasmid, amplified only one of the strands as single-stranded plasmid. The template strand should, however, have been digested by the selection enzyme making it very much less likely to be propagated in the bacteria. The resulting colonies were then screened by sequencing mini preps. By carrying out a control reaction with Bluescript plasmid and oligos (supplied with kit), the success of the SDM reaction could be analysed via blue / white colony selection on agar plates containing X-gal and IPTG.

Oligos used for SDM had to be HPLC purified before use. Also important for the reaction efficiency was the choice of tube used for the XL1-Blue heat shock transfection.

• Labelling of DNA Fragments

•Radioactive: For detection of membrane-blotted RNA, ^{32}P - labelled probes were prepared using the Random Primed Labelling kit from Boehringer MA. A reaction mixture consisting of 50 - 100 ng DNA, a mixture of random 6 nucleotide primers, 3 "cold" dNTPs and one ^{32}P - labelled dNTP, and dH_2O was heated to de-nature the template DNA. The temperature was then dropped to 4°C to prevent re-naturation. Klenow fragment (DNA polymerase) was added to the mixture which was then incubated at 37°C for 20 - 30 minutes, until the polymerization was complete. The mixture was then passed through a G50 column to separate the extended oligos (labelled) from the non-incorporated free nucleotides. The smaller molecules are trapped in the resin's beads while the larger molecules move between and through the beads, exiting the column first.

The success of the labelling reaction was determined by counting $1\ \mu\text{l}$ of column eluate using the ^{32}P program (Chrenkow counts) in a beta counter. A specific activity of over 10^8 cpm per μg DNA was required for good, specific RNA detection.

•Non-radioactive: For detection of RNA molecules *in situ* a labelling reaction was carried out which incorporated biotinylated dUTP nucleotides. In this case the DNA was labelled by nick translation instead of random primer. $2\ \mu\text{g}$ of plasmid DNA was incubated with dATP, dCTP, dGTP (each $50\ \mu\text{M}$) biotinylated dUTP ($50\ \mu\text{M}$), DNase I ($50\ \mu\text{g/ml}$), NT buffer ($50\ \text{mM}$ Tris pH 8, $5\ \text{mM}$ MgCl_2 , $50\ \mu\text{g/ml}$ BSA) and *E. Coli* DNA Polymerase I (20 units) for 2 hours at 15°C and then placed on ice. During this reaction, the DNase I makes single-stranded "nicks" in the template DNA. The polymerase then begins to re-synthesize a new strand at this point and continues until the polymerase comes to a nick in the template strand. Here the amount of DNase I is critical as it determines the average

length of the labelled DNA molecules. A portion of the reaction can be run on a 1% agarose to determine whether or not the reaction has to be extended further or not.

- **Preparation of RNA from Eukaryotic Cells**

Two different procedures were used to isolate two different types of RNA molecules. The "Total RNA" preparation was used to isolate all types of RNA from any cell type. This preparation worked best when the number of cells was not too high (did not exceed 5×10^6). When using total RNA to examine expression of some specific gene, it is important to remember that only a small percentage (approx.1%) of the total RNA is messenger RNA (mRNA) which is being used to express proteins. For this reason, a second preparation was used which was specific for mRNA molecules. As this process yields only mRNA molecules, it is best to start with a very large number of cells (more than 5×10^6).

- Total RNA Preparation. There are a wide range of total RNA kits available. The RNA-Clean kit (Qiagen) was used. It consists of only one solution, containing guanidinium thiocyanate and phenol. This is used either to resuspend a pellet of non-adherent cells or to scrape adherent cells down into. Cells were instantly lysed upon contact with this solution, the guanidinium thiocyanate protecting the RNA molecules from RNase degradation. Large numbers of non-adherent cells were difficult to resuspend from a solid pellet because of the instantaneous cell lysis. In these cases the pellet was partially resuspended with a small volume of PBS buffer. 200 μ l of RNA-Clean buffer was used per million cells and the cells pipetted up and down to ensure good lysis. Chloroform was added (200 μ l per 2 ml of homogenate), the samples vortexed for 15 seconds and then incubated on ice for 15 minutes. The samples were then centrifuged at 13000 rpm in an eppendorf centrifuge for 15 minutes. The upper phase was transferred to a fresh eppendorf tube and precipitated with 1 volume of isopropanol. This was incubated for 15 minutes on ice and then centrifuged for 15 minutes at 13000 rpm, at 4°C. The supernatant was then removed and 800 μ l 70% ethanol added to wash the pellet. This was repeated until no trace of phenol remained. Following the final wash, the pellet was briefly dried under vacuum and resuspended in 0.5% SDS or in 1 mM EDTA (pH 7). These solutions were prepared using DEPC- treated distilled water. OD₂₆₀ readings were not always accurate because of the presence of polysaccharides in the final RNA solution. The amount of polysaccharide depends on the cell type and even the stage of cell differentiation. To remove polysaccharide molecules, the precipitated and washed RNA pellet was completely resuspended in 200 μ l of "RNA-Clean Extension" solution. The polysaccharide was preferentially dissolved allowing the RNA to be collected via

centrifugation (13000 rpm for 10 mins). This was then resuspended in SDS or EDTA as described above.

- **mRNA Preparation.** Initially, cells were washed twice with ice-cold PBS (magnesium- and calcium-free). They were then scraped down into 2.5ml (per 10cm petri dish), or resuspended in 1 ml (per 2×10^7 non-adherent cells) of STEPS buffer (0.1 M NaCl, 20 mM Tris.HCl pH 7.4, 10 mM EDTA, 200 μ g/ml proteinase K, 0.5 % SDS) and transferred to 50 ml Falcon tubes. The cells were homogenized using an "Ultra turrax" for 1 minute at speed "blue" and incubated at 50°C for 20 minutes to accelerate protein degradation. Next, the NaCl concentration was increased to 0.5 M and 25-50 mg of oligo d(T)- cellulose added. This was then allowed to incubate at room temperature with shaking, overnight to allow the mRNA molecules to bind to the oligo d(T)- cellulose (The shaking should be sufficient to prevent the cellulose from sedimenting). The following day, samples were centrifuged (3 minutes at 2500 rpm), to pellet the cellulose. The supernatants were discarded, the pellets washed once, and then resuspended again in 10 ml Binding Buffer (0.5 M NaCl, 20 mM Tris.HCl pH 7.4, 10 mM EDTA, 0.2 % SDS). This was then loaded onto a filter column, the binding buffer passing through the filter leaving the mRNA-containing cellulose above the filter. The cellulose was then washed with approx. 60 ml of Wash Buffer (0.1 M NaCl, 10 mM Tris.HCl pH 7.4, 1 mM EDTA, 0.2 % SDS). Five 1 ml additions of Elution Buffer at 37°C were then made and the eluted mRNA collected in a 12 ml Greiner tube. A 300 μ l aliquot was removed for A₂₆₀ and A₂₈₀ spectrophotometric analysis. The NaCl concentration of the main RNA eluate was increased to 0.5 M and 2.5 volumes of 100 % ethanol are added. This was allowed to precipitate at -20°C overnight prior to centrifugation at 10000 rpm (Sorvall RC-4 centrifuge in a HB-4 swing-out rotor) for 30 minutes at 0°C. The supernatant was removed and the pellet briefly air-dried and resuspended in the required volume of dH₂O or 0.2 % SDS ready for gel loading.

- **Northern Blotting of RNA and labelled Probe Hybridization**

To the RNA samples (8 μ l) was added 4 μ l of 10% MOPS, 20 μ l formamide and 8 μ l of formaldehyde (stock conc. = 37%). Samples were incubated at 60°C for 5 minutes, transferred immediately to ice and run on a 1% agarose gel containing 20 mM MOPS, 1 mM EDTA, 5 mM NaAc and 6.6 % formaldehyde in running buffer consisting of 20 mM MOPS, 1 mM EDTA, 5 mM NaAc and 6.6 % formaldehyde, at 100 Volts. After the first hour, the gel was rotated 180° and the electrodes reversed. After the second hour, the buffer was replaced.

Following electrophoresis, the gel was removed and allowed to shake in 50 mM NaOH, 10 mM NaCl for 20 minutes. This acts to break the RNA molecules up into smaller fragments

which allows an efficient transfer to the support membrane. The gel was then washed with shaking in 20x SSC (3M NaCl, 0.3M sodium citrate pH 7) buffer 3 times, each 15 minutes. The RNA fragments were transferred (blotted) to a positively-charged nylon "Genescreen" membrane overnight using 20x SSC as transfer solution. The following day, the membrane was washed in 2x SSC to remove any contaminating agarose, dried and baked for 2 hours at 80°C under vacuum. This ensures that the RNA fragments are firmly attached to the membrane.

Prior to the addition of ^{32}P labelled probe, the membrane was "pre-hybridized" in Church buffer (0.5 M NaHPO_4 pH 7.2, 1% BSA, 1 mM EDTA, 7 % SDS) for between 15 minutes and 2 hours with shaking (or rotation). This buffer was removed and replaced with 7-10 ml of fresh pre-warmed (65°C) Church buffer. To this was added the labelled DNA probe following after having been heated to 95°C for 5 minutes and cooled on ice. The hybridization process at 65°C with shaking was continued overnight. The following day, the membrane was washed 3 times for 15 minutes in wash buffer (1 % SDS, 1x SSC) at 65°C and once in stringent wash buffer (1% SDS, 0.2x SSC) at 65°C. In this way non-specifically bound labelled probe should be removed. The membrane was wrapped in cling-film and placed in an autoradiography box with a film to expose for 60 minutes. This film was then developed to determine whether the membrane had to be washed further to remove excessive background or needed a longer exposure time.

• cDNA Synthesis for RT-PCR

In order to study gene expression at the RNA level via RNA prep and Northern blot, a large number of cells are required. In situations where too few cells are available or a quick check (before Northern analysis) is necessary, it is possible to use the PCR reaction to specifically amplify the "transcript" of interest. Because PCR can not be performed using RNA molecules as template, DNA copies of the RNA molecules must first be made. This is done by using the Reverse Transcriptase (RT) enzyme from Murine Leukaemia Virus to extend an oligo(dT) primer which hybridizes to the poly(A) tail at the 3' end of all processed mRNA molecules. cDNA molecules are thus extended until the cap structure at the 5' end of the transcripts is reached. These cDNA molecules can then be used as template in a standard PCR.

cDNA (and the subsequent PCR) was made using the GeneAmp[®] kit from Perkin Elmer. 1 µl of total RNA was added to a reaction mixture containing; MgCl_2 (5mM), 1x PCR buffer II, 2 µl DEPC treated water, dATP, dCTP, dGTP and dTTP (each 1mM), RNase inhibitor (1U/µl), MuLV Reverse Transcriptase (2.5 U/µl), Oligo d(T)₁₆ (2.5 µM). The total volume was 20 µl. This was allowed to incubate for 10 minutes at room temperature allowing the

oligo d(T) to anneal and partially extend. The reaction was then incubated at 42°C for 15 minutes for complete reverse transcription. The RNA-DNA complexes were de-natured upon incubation at 95°C for 5 minutes and placed immediately on ice.

Prior to PCR, 78 µl of a mix containing 2 mM MgCl₂, 1x PCR buffer II and 2.5 Units/100 µl AmpliTaq® DNA polymerase and 1 µl of each primer (final concentration of 0.15 µM) was added and the 100 µl mixture gently mixed. A single drop of mineral oil was added and the tube placed in a thermocycler (Stratagene Robocycler). For cycle conditions - see standard PCR method.

• Sterile Cell Culture Techniques

Prior to starting work, the UV lamps were switched off, and the air-flow system switched on and allowed to equilibrate. Then the front panel was then raised to the 20 cm mark. Latex gloves were worn at all times. These were sprayed with ethanol and allowed to air-dry before medium bottles etc were picked up. Before bottles of culture medium, pipette holders etc were placed into the hood, they were first sprayed with ethanol and allowed to air dry inside the hood. A cell culture bunsen burner was kept at a low yellow flame for the entire working time. Before opening and after closing sterile bottles and other containers, the the opening was briefly flamed (blue flame) without being allowed to become too hot. Sterile glass pipettes were used for almost all pipetting, these were passed through the flame before use. Disposable plastic pipettes were used when pipetting virus-containing media. Bottles or tubes with sterile contents were not left open for longer than necessary, the tops being held in the hand when possible. All waste which, during the course of work, became contaminated with media, cells or supernatants was autoclaved. After finishing work, all items were removed from the hood. The bunsen was turned off, the stainless steel and glass surfaces were sprayed with ethanol and wiped dry. The door was closed, the UV lamps were switched on and the air flow switched off.

• Passaging ("Splitting") of Adherent and Non-adherent Cells

Adherent cells were first washed once with sterile PBS medium (1.3 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ pH 7.4). Depending on the level of adherence, there was then added either PBS containing 5 mM EDTA (for normally adherent cells) or PBS containing 5 mM EDTA and 0.25% Trypsin (for highly adherent cells). The cells were examined under the microscope for signs of cells becoming less adherent (rounding up). Before the cells started coming away from the plastic, the PBS was removed via aspiration and the cells taken up in 10 ml of culture medium. For adherent cells this was DMEM with

either 5% or 10% FCS added. The cells were pipetted well to obtain single cells before a 10 μ l aliquot was removed for cell counting using a hemocytometer. COS7 cells were split 1:10 every three days. Mouse fibroblast cell lines were split 1:5 every two to three days. The frequency and density of splitting depended upon the growth characteristics of the individual cell lines. Lines which tended to form colonies could not be split too sparsely or left too long without being split again. The required number or portion of cells was then pipetted into a new culture bottle, which then had extra, fresh DMEM added to it.

Non-adherent cells were either grown in medium-sized culture bottles or in large (2L) roller bottles. After a 10 μ l aliquot had been counted, the required volume of cell-containing medium was removed and centrifuged at 1600 rpm in a Heraeus centrifuge for 5 minutes. The supernatant was carefully aspirated off and the cell pellet resuspended in fresh culture medium, which for non-adherent cells was typically RPMI with 5% FCS added. It was not always necessary to centrifuge a portion of cells down and resuspend then again in fresh medium, but this did help to remove any dead cells which do not sediment as quickly as living cells.

For both processes, the PBS and culture media were pre-warmed to 37°C to avoid heat shocking the cells. Cells were grown in incubators at 37°C with 6% CO₂.

• Freezing and Thawing of Cells into and out of Liquid Nitrogen

Before being frozen, cells were kept continuously in growth phase. 5 million cells were centrifuged down at 1600 rpm for 3 minutes and resuspended in 500 μ l of FCS containing 10% DMSO. This was swiftly transferred to a pre-cooled (4°C) freezing tube and placed immediately at -70°C. After 2 days at -70°C the tube was then moved quickly to liquid nitrogen (-180°C) storage. DMSO in solution is extremely toxic for the cells, thus it was often preferred to first pipette the DMSO into the freezing tube and then to add to this the cells in FCS.

When thawing cells, it was very important that the thawing time be minimized. The frozen tube was transferred from liquid nitrogen to a 37°C waterbath until its contents had thawed. The contents of the tube were then mixed with 10 ml of fresh medium and centrifuged at 1600 rpm for 5 minutes. The supernatant was then removed and fresh medium added and used to resuspend the cells. These were then transferred to a medium-sized culture bottle and incubated at 37°C.

- **Calcium Phosphate Transfection of Adherent Cells**

20 hours before transfection, cells were seeded into 10cm petri dishes at a density which ensured that after 3 days of normal growth, the cells would again be ready for splitting. For COS7 cells, 3×10^5 cells were seeded whereas for Hela cells, 5×10^5 were required. For Fluorescence *in-situ* hybridization analysis (FISH), COS7 and Hela cells were seeded at densities of 7.5×10^4 and 10^5 cells per microscope slide, respectively. 4 hours before transfection, the medium (DMEM 10% FCS) was changed.

The DNA (20-30 μ g) was pipetted into a 12 ml plastic tube with 50 μ l of sterile 2.5 M CaCl_2 and made up to 250 μ l with sterile dH_2O . To this was added (dropwise) 250 μ l of 2x BES buffer (10.7 g/L BES, 250 mM NaCl, 2 mM Na_2HPO_4 pH 6.96). The 2x BES buffer was added using a P200 pipette (Gilson), held in one hand whilst a Sarstedt pipette-aid and pasteur glass pipette were held in the other and used to bubble air through the solution from the bottom of the plastic tube. This was found to result in a very fine precipitate (visible using a light microscope) upon mixing of the two solutions.

This solution was incubated at room temperature for 30 minutes with occasional gently mixing and then pipetted (dropwise) on to the plated cells. Fresh medium was not added. Cells were incubated for 12-14 hours at 37°C and then washed well with sterile PBS to remove as much precipitate as possible. Fresh medium was added and the cells allowed to incubate again at 37°C for up to 2 days or until confluent.

- **DEAE-Dextran Transfection of Non-adherent Cells**

10 million cells per transfection were centrifuged down in 250 ml Corning centrifuge bottles at 1600 rpm for 10 minutes at 4°C . The supernatant was removed via aspiration and 2 ml of TBS buffer (25 mM Tris pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $\text{CaCl}_2 \cdot (\text{H}_2\text{O})_6$, 0.5 mM $\text{MgCl}_2 \cdot (\text{H}_2\text{O})_6$, 0.6 mM Na_2HPO_4) added per transfection. After being carefully resuspended in TBS, the cells were aliquoted (2ml) into 5 ml plastic tubes. These were then centrifuged in a table top centrifuge for 2 minutes at 2000 rpm to pellet the cells. The supernatant was again removed via aspiration.

The DNA to be transfected was pipetted into a 5 ml plastic tube. The volume was made up to 250 μ l with TBS buffer. To this was added a 250 μ l mixture of TBS containing 1 mg/ml DEAE-dextran and 40 μ g/ml chloroquine. This 500 μ l volume was used to resuspend the cell pellet. After a 30 minute incubation at room temperature with occasional gently mixing, 2 ml of fresh TBS was added to each tube and the tubes centrifuged again to pellet the cells. The pelleted cells were then resuspended in RPMI medium (5% FCS) and transferred to a medium-sized culture bottle. The cells were incubated for 48 hours before being harvested and processed further.

N.B. The chloroquine used was taken from a freshly prepared 4 mg/ml stock solution. Chloroquine acts to reduce endosomal degradation of the endocytosed DNA-dextran complex.

• **Electroporation of Eukaryotic Cells**

Sterile electroporation cuvettes with a 0.2 cm electrode gap were pre-cooled on ice. The DNA (20-30 µg) was precipitated in 0.15 M NaAc and 2 volumes of 100% ethanol. After washing with 500 µl of 75% ethanol, the DNA was resuspended in 20 µl of sterile PBS. Cells were washed once in PBS and then centrifuged and resuspended so that 180 µl of PBS would contain 2 million cells. The DNA was added to this and the mixture carefully pipetted and transferred to the pre-cooled cuvettes, on ice.

The electroporation apparatus (Bio-rad Gene Pulser) was connected to the capacitance controller (Bio-rad) and set to : 0.26 KV, 960 µF with "external" selected. The cuvette holder was connected to the capacitance controller (external). The contents of the cuvette were then briefly mixed and the cuvette placed into the holder. The cells were pulsed and the cuvette placed on ice for 10 minutes before the cells were taken up in fresh medium and transferred to a culture bottle and incubated at 37°C. When transferring the electroporated cells to the culture bottle, the large mass of dead (lysed) cells was removed using the end of a blue pipette tip. Presence of too many dead cells in the fresh medium inhibits the growth of surviving cells. The electroporation protocol was optimized for use with COS7 cells - resulting in an efficiency of approximately 40 %, assayed via X-Gal staining of cells transfected with a β-galactosidase expressing plasmid. Approximately 50 % cell death occurred at the optimum conditions.

N.B. It is also possible to use medium instead of PBS as the electroporation medium - in the case of COS7 cells this was not advantageous. Cuvettes were washed after use with distilled water, then 1% SDS, then water again and finally 75% ethanol. They were then stored under 75% ethanol at room temperature.

• **β-Galactosidase Expression Assays**

Three different assay methods were used. (i). The standard assay, in which the cells were fixed (and therefore died) involved the use of the X- Gal (5-Bromo-4-Chloro-3-indolyl-β-D-galactoside) substrate. Here the cells were washed once in PBS and fixed using ice-cold fixing solution (2% formaldehyde and 0.2% glutaraldehyde in PBS). The cells were incubated for 10 minutes in fixer at 4°C and then washed three times with PBS to remove the fixer. Pre-warmed (37°C) staining solution (5 mM potassium ferrocyanide, 5 mM

potassium ferricyanide, 2 mM MgCl₂ in PBS) with 100 µl X- Gal (stock 20 mg/ml in Dimethylformamide) per 4 ml added, was added to the fixed, washed cells. This was incubated at 37°C overnight. Depending on the level of expression, it was often possible to observe blue staining after 2-3 hours. The X- Gal aliquots were stored at -20°C in darkened tubes until required.

(ii) When it was necessary to keep the stained cells alive, for example to sort via Fluorescence Activated Cell (FAC) sorting, the substrate FDG was used. In this protocol, cells were washed once with sterile PBS and then resuspended in PBS at a concentration of 10 million cells per ml. To this was added chloroquine to a final concentration of 300 µM and the mixture put on ice. A 100 µl (10⁶ cells) aliquot was then removed and incubated at 37°C for 20 minutes. 100 µl of pre-warmed FDG (in dH₂O) was then added and quickly and mixed well. This was incubated at 37°C for 60 seconds only. During this step, the FDG was taken up by the cells as they swelled due to osmosis. To prevent cell death, 1.8 ml of ice-cold isotonic solution (1 µg/ml propidium iodide in PBS) was added. The cells were then put back on ice for 5 minutes. PETG (1mM final conc.) was then added to stop the FDG leaking back out of the cells. The cells were then analysed using a FAC scan machine. Cells able to convert the FDG (and thus containing the β-galactosidase protein) were fluorescent. Propidium iodide allowed the visualization of the dead cell population. By selecting the population of cells that were FDG positive and PI negative, a readout of percent positive cells was obtained. This "gated" area was then sorted to give only the live, transfected cells. These cells were put back in culture, expanded and assayed again until a 100% transfected population was obtained.

(iii) Plasmids expressing the Lac Z gene were co-transfected and assayed as in internal control for transfection efficiency. This was done on occasion as part of the assay for Chloramphenicol Acetyltransferase (CAT) protein. In this situation, to a portion (30 µl) of the lysate derived from transfected cells was added 500 µl assay buffer and 100 µl of ONPG (2 mg/ml). This was incubated at 37°C for 2-3 hours (or until a yellow colour became visible). At this point 250 µl of 1 M Na₂CO₃ was added to stop the reaction. The optical density was then measured at 420 nm.

• Chloramphenicol Acetyltransferase (CAT) Expression Assay

Transfected cells were harvested after 48 hours incubation at 37°C and washed once with PBS. The cell pellet was resuspended in 100 µl of 250 mM Tris (pH 7.4) in a "safe-lock" eppendorf tube. The cells were then freeze-thaw lysed by placing the tube in liquid nitrogen for 2 minutes and then at 37°C for 5 minutes. This was repeated three times. The tube was then centrifuged at 14000 rpm at 4°C for 5 minutes. The supernatant was then

transferred to a fresh eppendorf tube. Either a β -galactosidase assay was carried out (if a LacZ plasmid had been co-transfected) using 30 μ l of supernatant or a total protein assay, using 1 μ l supernatant. To determine total protein content, the Bio-rad Protein Assay solution was diluted 1:5 and added to the 1 μ l supernatant. The optical density at 595 nm was then measured. The remaining supernatant was incubated at 65°C for 10 minutes, then centrifuged at 14000 rpm for 5 minutes at 4°C. Again the supernatant was pipetted into a fresh eppendorf tube and placed on ice.

A reaction "cocktail" was then prepared which consisted of 4 μ l/sample ^{14}C -Chloramphenicol, 0.67mM Acetyl CoA, 250 mM Tris pH 7.5 and dH₂O up to a volume of 120 μ l per sample and kept on ice.

After analysis of the OD₅₉₅ values (or OD₄₂₀), an equal amount of total protein containing (or β -galactosidase expressing) lysate was pipetted into a fresh eppendorf tube for each sample. The volumes were standardized such that the lysate of lowest activity had a volume of 30 μ l and from all others a smaller volume of lysate was taken. The difference in volume was compensated for by addition of 250 mM Tris up to the 30 μ l volume. To the 30 μ l volumes was then added 120 μ l of "cocktail". The tubes were then mixed carefully and incubated at 37°C for 60 minutes. 800 μ l of ice-cold ethylacetate was then added to each sample. This was vortexed well and then centrifuged at 13000 rpm for 10 minutes. The upper layer was then transferred to a fresh eppendorf tube and dried in a vacuum drier for 45 minutes. 30 μ l of cold ethyl acetate was then added and the tube shaken in an eppendorf shaker for 10 minutes to ensure good solution of the chloramphenicol desiccate. 30 μ l samples were then loaded onto a thin layer chromatography sheet which was then placed in a chromatography tank containing a mixture of chloroform (190 ml) and methanol (10 ml). This mixture had to be prepared at least 2 hours in advance to allow equilibration of the solvent mixture. Normally it was possible to re-use the solvent at least 5 times. When the solvent front had run about 90 % of the TLC, the TLC was removed, allowed to air dry and was then placed in an autoradiography box. A film was then placed inside and allowed to expose overnight.

Films were then scanned using an Apple Macintosh computer and Adobe Photoshop version 3.0 . Regions of interest were selected and then copied into Deneba's Canvas version 3.5 program, where they were further processed (addition of sample names and acetylation values). Acetylation values were measured using a ^{14}C -counter (Berthold Tracemaster 20 automatic TLC-linear analyzer) which scanned the TLC in the vertical direction starting from the loading point. The number of counts per second at different positions along the vertical line was then plotted. This way values for non-acetylated and the different mono-acetylated forms of ^{14}C Chloramphenicol could be obtained and a percentage acetylation calculated.

- **Fluorescence *in-situ* Hybridization (FISH)**

This technique was used to visualize specific RNAs in the two main cellular compartments (nucleus and cytoplasm). First, cells were transfected via calcium phosphate precipitation on poly-L-Lysine (4mg/ml) coated "Superfrost" microscope slides in 4-chamber "Quadriperm" tissue culture plates.

Fixing & Permeablizing of cells: Cells were washed three times with PBS and then fixed in 4% paraformaldehyde (freshly prepared) for 20 minutes at room temperature. After another wash in PBS, cells were partially permeabilized by incubation in STPBS (0.5% saponin (w/v), 0.5% Triton X-100 (v/v) in PBS) for 20 minutes at room temperature. The cells were then washed once more in PBS and then incubated in 20% glycerol for at least 30 minutes. Slides were then removed, frozen in liquid nitrogen. If not being directly further processed then the slides were stored at -70°C. If continuing, then the cells were removed from the liquid nitrogen and allowed to thaw very slowly at room temperature before being placed in PBS prior to the denaturation step.

Labelling, precipitation & denaturation of probe DNA: Construct J258 was used to prepare a CAT probe and the CMV-LacZ plasmid was used to prepare a LacZ probe. Probes were labelled by nick translation using biotin-11-dUTP or Digoxigenin-11-dUTP in place of dTTP (see non-radioactive labelling). The average length of the probe molecules (200 nt) was checked on a 1% agarose gel (containing ethidium bromide). Probe DNA (200-400 ng per slide) was precipitated together with 7µg of salmon sperm DNA, 3µg of human COT1 DNA by 0.05 volumes of 3M sodium acetate and 2 volumes of 100% ethanol. This was incubated at -80°C for at least 30 minutes before being pelleted via centrifugation at 13000 rpm in an eppendorf centrifuge for 15 minutes. The pellet was washed twice with 500 µl of 70% ethanol and allowed to air-dry. It was then resuspended in a solution of 50% deionized formamide, 2x SSC, 10% dextran sulphate and 50 mM sodium phosphate.

The probe mix was denatured at 75°C for 5 minutes and then placed at 37°C to allow the COT1 DNA to anneal to any repetitive (GC rich) sequences.

Denaturing of cells: Cells were denatured in 40% formamide, 2x SSC (pH 7.0) at 73°C for 3 minutes. At this concentration of formamide, only RNA molecules are de-natured (not DNA). To verify that signals seen were not from DNA, an RNase digestion was made in parallel (100µg/ml RNase A).

Hybridizing & washing: The denatured probe and cells were then incubated together (by placing the coverslip (containing probe) on top of the cells (on the slide)) at 37°C for at least 12 hours in a humidified chamber to prevent probe evaporation. The following day, the slides were washed three times (each 5 minutes) with low stringency buffer (50% formamide, 2x SSC, pH 7.0 at 42°C) and then three times (each 5 minutes at 42°C) with

high stringency buffer (0.1x SSC pH7.0 at 60°C). Cells were then incubated in a solution of 4x SSC containing 4% BSA for 20 minutes at room temperature.

Detection of probe: Probe was detected using either fluorescein conjugated avidin or fluorescein conjugated sheep anti-digoxigenin antibody. This was carried out using a solution containing 4x SSC and 1% BSA. A 10 minute incubation with propidium iodide (2 µg/ml) was made to enable visualization of cell nuclei.

Analysis of staining: Confocal laser scanning images were acquired on a Zeiss confocal laser scanning microscope (LSM 310) equipped with an argon ion laser and a helium neon laser using an oil immersion 63x plan-APOCHROMAT lens (Zeiss). Red (propidium iodide) and green (fluorescein) images were simultaneously obtained by excitation of rhodamine and fluorescein at 488 nm.

SECTION A: Sub-Cloning of HTLV-I based Constructs

• Cloning of the LTR_{JAY} vector

Initially, a vector (LTR_{JAY}.HCB.LTR) was constructed using plasmids from two sources: (i) The pHTLV-tat I construct (Nerenberg *et al.* 1987) and (ii) Derivatives of pHTLV-tat I cloned by K. Sach (1991) which contained the Murine Leukaemia Virus (MLV) extended psi sequence and the pgk-Neo cassette, both from pHMB (Hawley *et al.* 1989; Bender *et al.* 1987). Fragments from these constructs were cloned together into the Bluescript II SK⁺ phagemid which contains a large number of unique restriction sites and allows the production of single-stranded DNA through the use of fd phage. The resulting construct was named J157. (NB. See Section D for flow diagrams of all cloning schemes.)

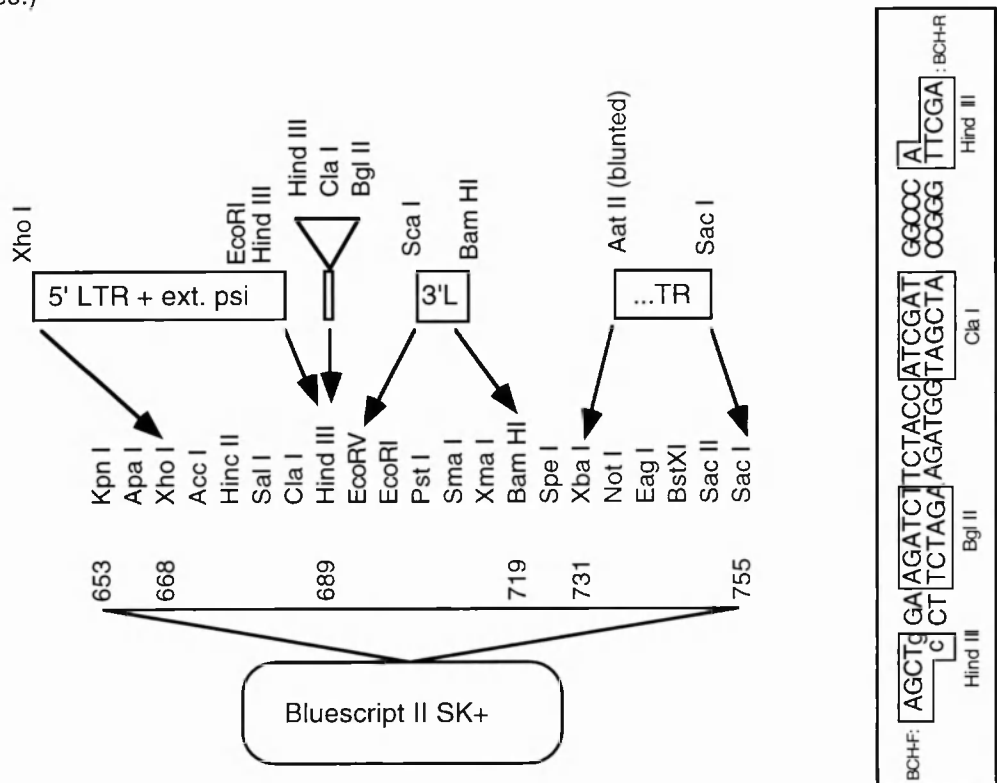


Figure 5. Cloning procedure for reconstruction of the LTR_{JAY} vector (J157) inside Bluescript II SK⁺. The 1.7 kb Xho I- Hind III 5'LTR-psi fragment was cloned first. This consisted of a fusion between the HTLV-I 5'LTR and the MLV extended packaging sequence (nt. 165 to 1037 of MLV sequence). Next the HTLV-tat I-derived 180bp ScaI-Bam HI fragment was cloned into EcoRV-Bam HI. This reconstituted the first part of the 3'LTR (up until the site of the old Aat II). The remaining 600bp was inserted as a blunted Aat II- Sac I fragment into the blunted Xba I-Sac I sites. This fragment was cloned from pUC18.tax.3'LTR after deletion of the BamHI-Bgl II fragment. Finally, two oligos (#11 and #12) were annealed to one another and inserted into the unique Hind III site. This resulted in the insertion of a Cla I and a Bgl II site as well as maintaining one of the Hind III sites - the other being mutated. The multiple cloning site orientation used was the Hind III-Cla I-Bgl II (HCB) orientation.

Construct J157 was sequenced, the 5'LTR sequence is shown below:

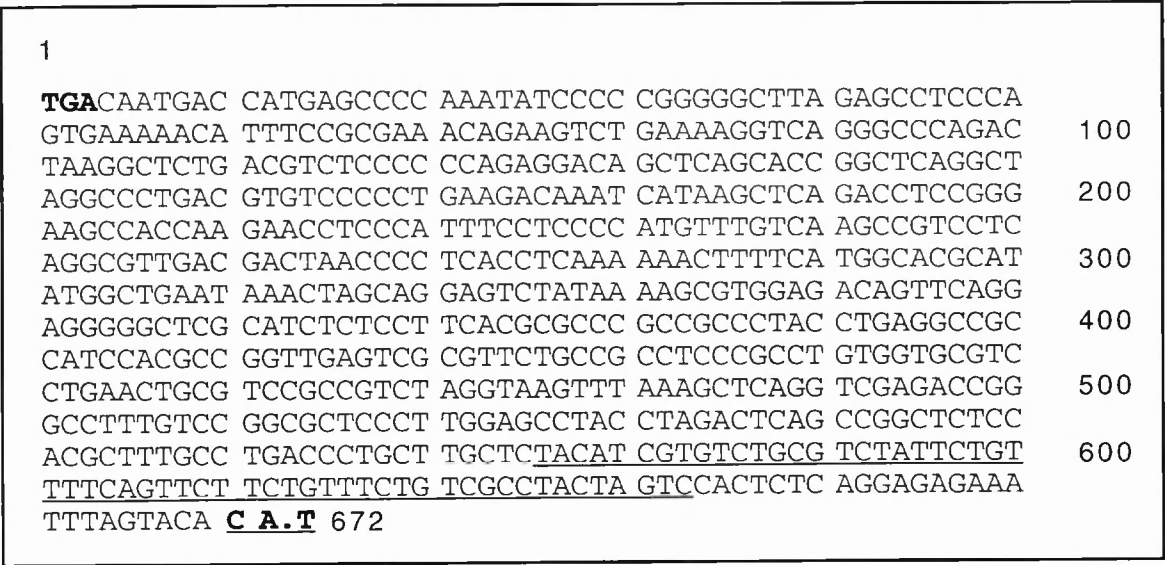


Figure 6. 5' LTR sequence of construct J157. The sequence extends from the 5' end of the U3 region (bold) to the integration region (bold, underlined) at the 3' end of the U5 region. The expected deletion inside the U5 region was seen to extend from bp 575 and continue up until bp 727 of the published sequence (Accession # J02029). A 58bp insertion of unknown origin was sequenced (underlined) and seen to be G-T rich in nature. A single "G" deletion was observed at the integration region, however, as infectious virus had been successfully produced from constructs with this deletion, it was thought unlikely to play an important role.

These LTR_{JAY} vectors have the CRS region (Seiki *et al.* 1990) deleted and should allow LTR-driven expression in a Rex-independent manner. The observed U5 insertion may have been made to reconstitute the poly A site. In addition to expression from the LTR_{JAY} constructs, we were also interested in expression from the "full length" LTR₇₅₅. It was therefore decided to insert a full length LTR in place of the mutated JAY LTR.

• Cloning of the Full Length LTR₇₅₅ vector

A second series of vectors was cloned which contained the full length (755bp) 5' LTR attached to the MLV extended packaging sequence (ext. psi). To this aim, both the new 5' LTR and the extended psi sequence were PCR amplified independently, using the 3' LTR from pHTLV tat1 and pHMB, respectively, as templates. Both PCR products were then used as templates in a third PCR reaction which resulted in "ligation" of the two fragments (fig.7).

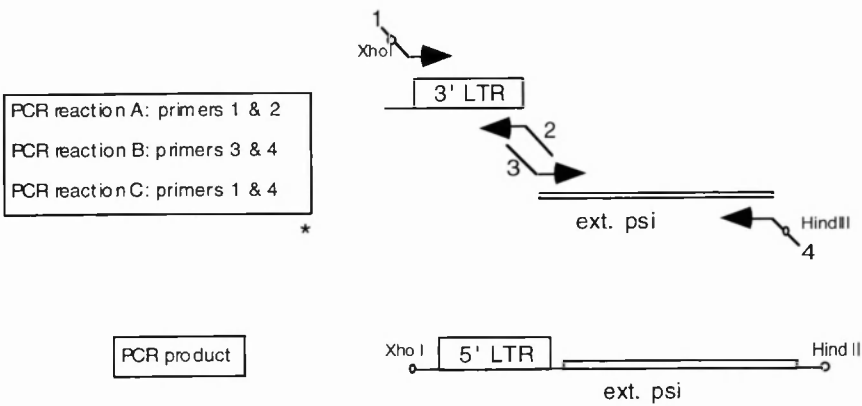


Figure 7: PCR construction of 755bp 5' LTR. Due to the nature of oligos 2 (#29) and 3 (#92), there was a 16bp overlap at the 5' ends. The maximum annealing temperature of the 16bp overlap was chosen to be 2° lower than that of the two amplifying oligos (63° and 65° respectively). This slight difference promoted the annealing of the two template molecules to each other before the reaction three amplifying oligos (#91 and #11) could anneal and thus maximized the yield of full length PCR product (1.7kb). *See Materials section for oligonucleotide sequences.

The Xho I - Hind III fragment was then cloned to produce two different constructs (fig.8).

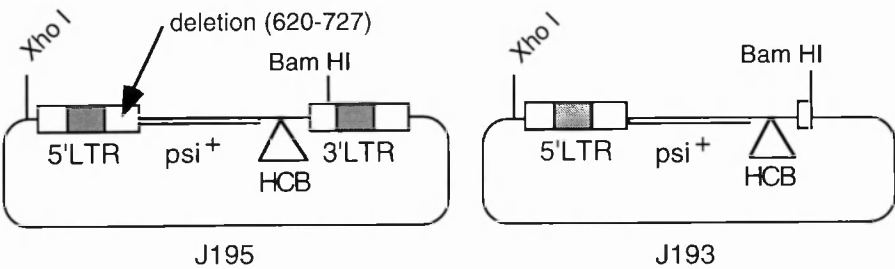


Figure 8. Cloning of the new LTR-psi fragment (Xho I- Hind III). This was done in construct J157 (to yield construct J195) as well as one of the cloning intermediates (J114a) to yield construct J193. See section D for details of cloning intermediates.

J193 was sequenced as there was no chance of the sequencing primer (#20) annealing to both LTRs. The sequencing showed that the deletion / mutations present in the JAY LTR were no longer present. DNA sequencing later revealed that the sequence of the PCR product cloned to create J195 was not the same as that of the first sequenced J193! While J193 contained the full length LTR₇₅₅, J195 contained a deleted LTR. The deletion was again seen to be present in the U5 region and it was suspected that the PCR "product" contained both full length (755bp) LTRs and U5-deleted (628bp) LTRs. The formation of secondary structure within the U5 region during the PCR could have produced such a phenomenon. Closer examination of the sequence of the J195 LTR showed that the oligos used in the PCR protocol (#91 & 29) were present and that the deletion was from bp 576-703. This removed the entire CRS region (described by Seiki *et al.* 1990) without disturbing the *Cis*-activating Sequences (CAS) (Kashanchi *et al.* 1993) which are required for basal expression from the HTLV-I LTR. The larger deletion present in LTR_{JAY} removed not only the CRS but also 45% of the CAS element. For this reason, the J195 mutant was used as a CRS⁻ LTR, termed LTR₆₂₈ in subsequent experiments.

A construct consisting of two 755bp LTRs (J225) was then cloned by inserting the 3'LTR into the J193 construct as a Bam HI - Sac I fragment. This Bam HI site was created by Kerstin Sach by cleavage and blunting of the existing Aat II site in the U3 region and ligation of Bam HI linkers.

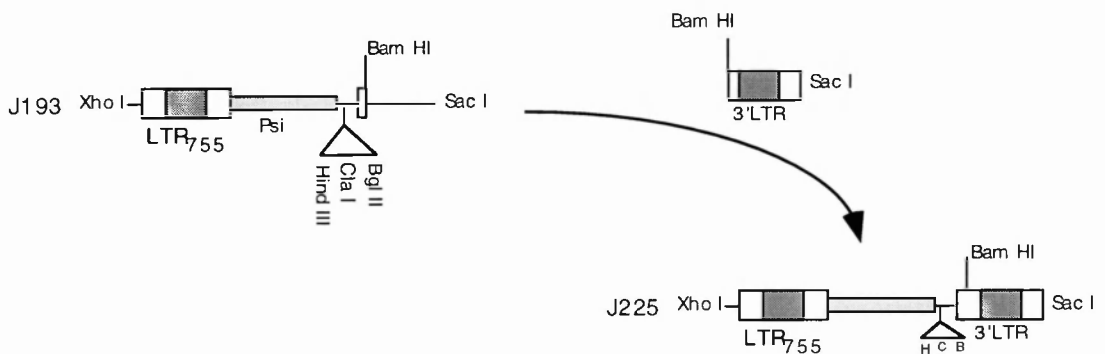


Figure 9. Cloning of construct J225. The Bam HI/Sac I fragment was removed from construct J195 and inserted into J193.

• Insertion of the PGK promoter-Neomycin Resistance Cassette - DC Vectors

The PGK- Neo cassette was derived from the pHMB construct. It was cloned as a BamHI fragment into the BamHI site in the 3'LTR .

By placing the second gene (Neo) inside the 3'LTR, it was thus hoped to overcome the phenomenon of promoter silencing.

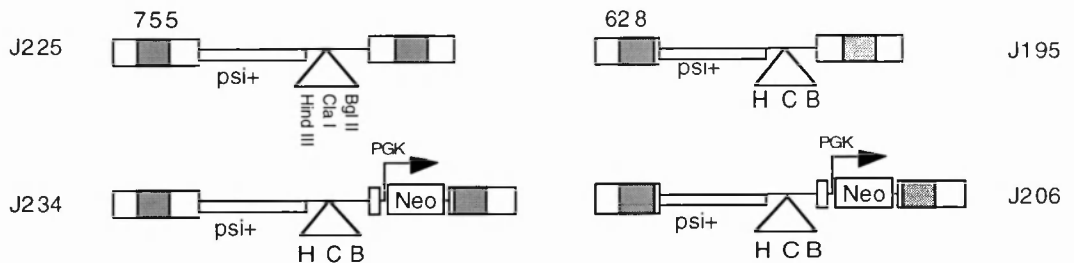


Figure 10. Insertion of the 1.35 Kb PGK-Neo Bam HI fragment to give DC (Double Copy) vectors having either a complete (755bp) 5'LTR or the CRS deletion (LTR₆₂₈).

• PCR cloning of the Chloramphenicol Acetyltransferase (CAT) reporter gene

To examine the expression levels from the two different LTRs a suitable reporter gene had to be chosen. The CAT gene offers a very sensitive readout of LTR expression which can be quantitated very accurately by performing a CAT assay. In the CAT assay, lysates of transiently transfected cells are added to a cocktail containing ¹⁴C-labelled Chloramphenicol and Acetyl Coenzyme A. The Chloramphenicol is acetylated by CAT enzyme from the lysates and then migrates more rapidly on thin layer chromatographs (TLCs). The relative percentage of acetylated Chloramphenicol can then be quantitated using a ¹⁴C detector.

A suitable CAT gene with the correct cloning sites was not available, so once again a PCR was performed. The primers (see materials for sequence) contained restriction sites for Hind III and Bgl II and amplified the CAT gene from the plasmid pBL-CAT2 (Luckow and Schütz 1987). The PCR was optimized to minimize mis-incorporations. Deep Vent[®] DNA polymerase (NE Biolabs) was used in a 16 cycle reaction with 4mM Mg²⁺. PCR products were cloned directly into the recipient plasmids (fig.11) and then sequenced using the PCR oligos (#101 & 102). For each of the four plasmids, two clones were sequenced. None of the eight clones sequenced had any mis-incorporations in the entire length (750bp).

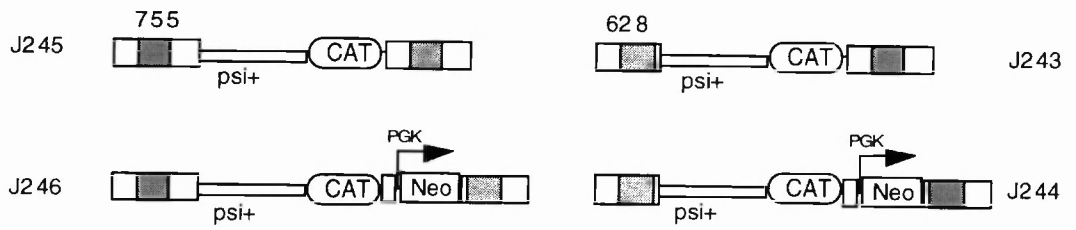


Figure 11. Double-copy and standard CAT expression vectors. After being PCR amplified, the CAT PCR product was digested with Hind III and Bgl II. This was then inserted into each of the four constructs pictured in fig.10 to yield the above constructs.

The resulting constructs (J243, J244, J245 & J246) were then used in transient transfections of Jurkat T cells together with plasmids expressing either Tax or Rex from a CMV promoter.

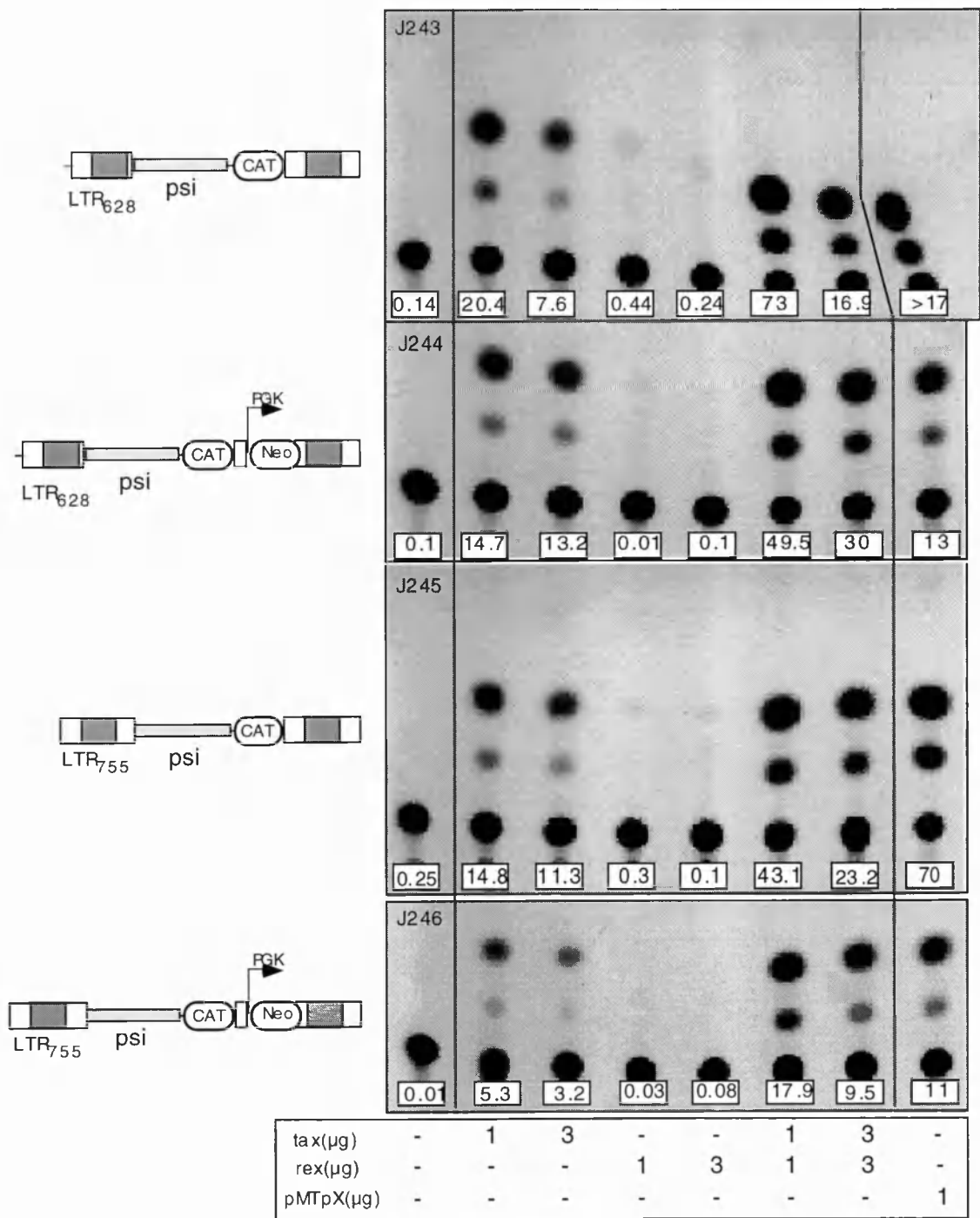


Figure 12. CAT expression Assay of single and double copy CAT vectors. Cell lysates were made from Jurkat cells 48hrs after DEAE transfection of 5μg of each CAT expression construct. CAT assays were performed using total protein concentration to standardize lysate volumes between samples. CAT expression was seen from both the CRS-containing and CRS-deleted constructs upon co-expression of Tax alone (J245 & J243, lane 2). Percent conversion of Chloramphenicol is shown for each reaction (boxed).

From this experiment it was clear that the PGK-Neo cassette insertion although not removing CAT expression, did reduce it. As constructs J245 and J246 contain the CRS, it was expected that CAT

expression from these constructs, relative to constructs J243 and J244, would be very much lower. Interestingly, the expected repression of CAT expression upon Tax transactivation alone was not observed in these LTR₇₅₅ constructs.

Optimizing levels of Tax and Rex *trans*-activation

The amounts of Tax and Rex appear to be critical - 1 µg of Tax plasmid together with not more than 1µg of Rex plasmid led to the best CAT expressions. This experiment was repeated with constructs J243 and J245 to determine the optimal amounts of Tax and Rex plasmids for co-transfection (see fig. 13).

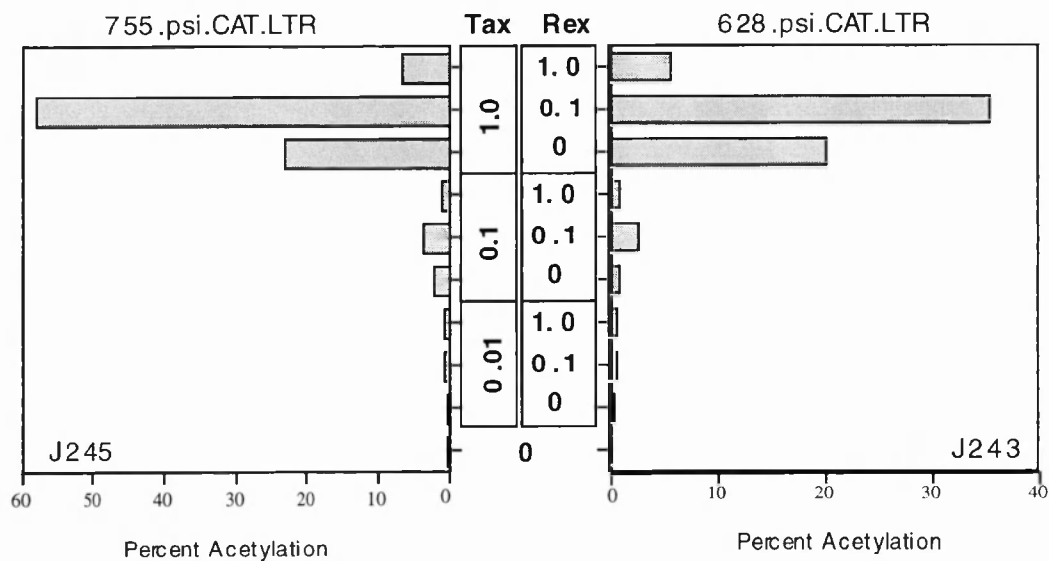


Figure 13. Optimization of co-transfection. 5µg of either J243 or J245 was transfected with increasing amounts of Tax and Rex expressing plasmid into Jurkat T cells. Lysates were made, standardized for total protein concentration and assayed for CAT activity.

This experiment clearly showed that the optimum amounts of Tax and Rex plasmid to be co-transfected with 5µg of CAT reporter were 1µg and 0.1µg, respectively. The reduction of CAT expression upon increasing levels of Rex plasmid was thought to be due to the fact that both the Tax and Rex expressing plasmids use a CMV promoter. The availability of transcription factors for CMV-Tax expression is reduced if the amount of CMV-Rex plasmid is increased (squenching (Gill and Ptashne 1988)). This was tested by transfecting increasing amounts of a control CMV plasmid (results not shown) and indeed, the level of Tax *trans*-activated CAT expression was reduced upon addition of plasmid CMV-ER (where ER is the cDNA for the hormone binding domain of the human Oestrogen receptor). The reduction in CAT expression upon addition of increasing levels of Rex is thus due to promoter competition and not Rex itself, and is ignored in subsequent experiments.

After characterization of the unexpected mutation within the U5 region of construct J195 it was necessary to re-check construct J245 by DNA sequencing to ensure that the CRS was still present. The DNA was complete. Detailed comparison of our constructs with those of Seiki & Yoshida - who have shown that constructs with two full length LTRs do not express upon Tax *trans*-activation alone - revealed that the only significant difference was the presence of the MLV extended packaging signal, in our vectors. This is because the constructs were initially designed with the aim of producing MLV pseudotyped HTLV-I based virus. It was decided to remove the psi sequence to test whether it had been responsible for the unexpectedly high level of expression.

• Removal of the MLV Extended Packaging Sequence

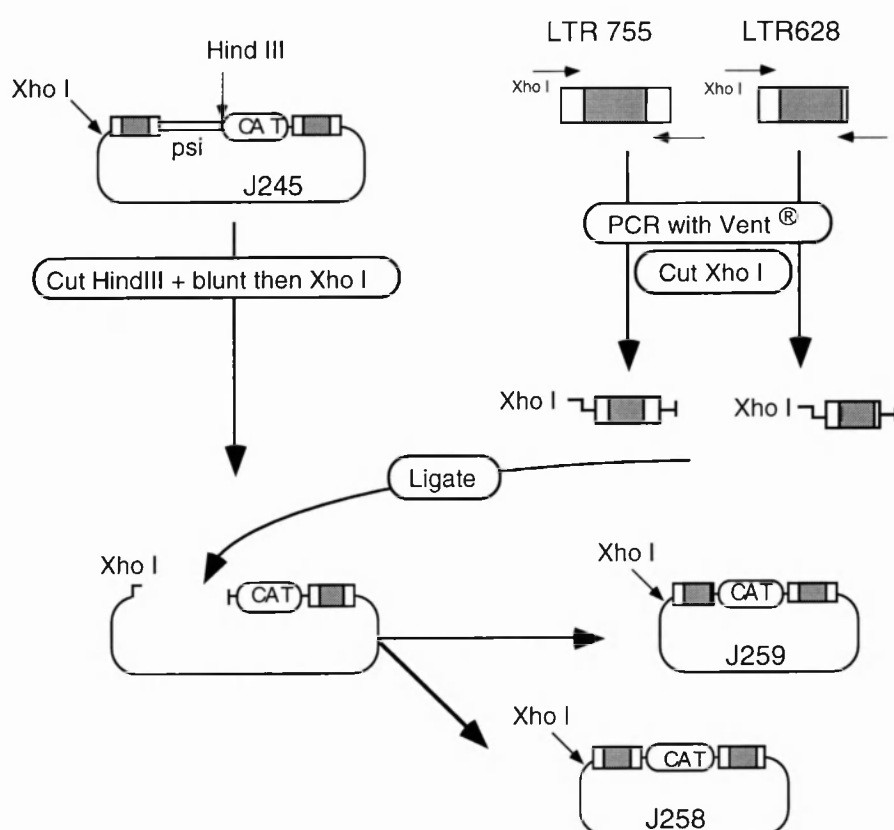


Figure 14. Deletion of the extended psi sequence. Removal of the 5'LTR-psi sequence from construct J245 via Hind III digestion, blunting and digestion by Xho I, was followed by Vent Polymerase PCR of Full length and CRS⁻ LTRs (The Vent polymerase used contained a proof reading activity and therefore produced a PCR product without 3' overhangs). These PCR products were then digested with Xho I to give one sticky and one blunt end, and ligated back into the prepared J245 vector backbone. This resulted in the destruction of the original Hind III.

Following a sequencing check (oligo #20) of the two newly amplified 5'LTRs, the constructs (J258 & J259) were used to transfect Jurkat T cells. Tax and Rex expressing plasmids were co-transfected. Lysates were made and assayed for CAT activity.

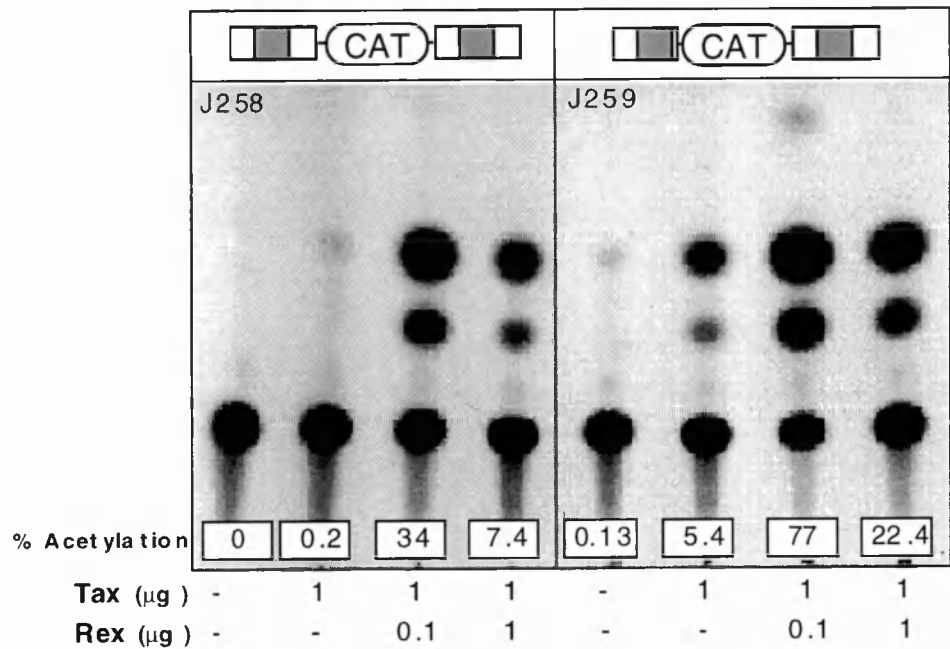


Figure 15: CAT expression assay of full length and CRS⁻ constructs following psi deletion. Lysates were made 48 hours after transfection with 5μg of CAT plasmid DNA, standardized for total protein and assayed for their CAT activities. Removal of the psi sequence allowed the CRS effect to be seen. Expression from construct J258 was repressed (lane 2) whilst expression from the CRS⁻ construct (lane 6) was significantly higher. Addition of Rex, however, led to a further increase in expression (lane 7).

Observation One: The HTLV-I LTR encodes a second negative regulatory element

Construct J258 was then seen to be highly Rex dependent (fig. 15 compare lanes 2 and 3). Upon expression of Tax, CAT activity was completely repressed. This was presumably due to the nuclear retention function of the CRS.

Tax *trans*-activation of construct J259 (CRS⁻) allows a low level of CAT expression, but is also highly Rex inducible. Although CAT expression increases to 5.4%, the repression present in J258 is not completely removed. After removal of the CRS, Rex was still able to give a 14-fold enhancement (lane 7).

The fact that removal of the CRS from the 5'LTR did not result in a complete removal of repression indicated that there was another negative element present in construct J259. It had recently been shown that the HIV Rev response element (RRE) contains a negative element (Brighty and Rosenberg 1994) in addition to the well characterized positive element. This element led to the retention of transcripts in the nucleus. For this reason we decided to look for a downstream negative element (DNE) inside the 3' LTR of HTLV-I, as this is where the HTLV-I positive element (RxRE) is situated.

Observation Two: MLV extended psi sequence overcomes the CRS-mediated repression

Removal of the MLV extended psi sequence from the two constructs, revealed a difference in Tax-induced CAT expression between constructs containing and lacking the CRS. Therefore, the psi sequence appeared to have overcome the nuclear retention of the full length construct and to enhance expression in the CRS⁻ construct. From MLV sequence data (Weiss *et al.* 1985), there were no known splicing signals present, suggesting that a positive element existed which was capable of promoting the transport of transcripts from the nucleus to the cytoplasm. Such a sequence termed CTE (constitutive transport element) had been recently described (Bray *et al.* 1994) to be present in the Mason-Pfizer Monkey Virus (MPMV). When inserted into a Rev-dependent HIV envelope expression plasmid, it resulted in the removal of Rev dependency. HIV *env* transcripts are normally trapped in the nucleus until HIV Rev protein binds to the transcript's Rev response element (RRE) allowing it to leave the nucleus.

The next series of experiments (SECTION B) was carried out in order to explain how the MLV extended psi sequence was able to remove the Rex dependency of the HTLV- I CAT constructs. Experiments aimed at locating the downstream negative element (DNE) will be described later in SECTION C.

SECTION B: Presence of a CTE inside the MLV Extended Packaging Sequence

• Re-introduction of Psi Fragments

Initially, it was important to determine whether the psi effect was due to a sequence specific element, - such as a CTE - or whether the effect seen was size dependent. RNAs with longer 5' non-translated regions may tend to be more stable (Muhlrads *et al.* 1994, 1995). To differentiate between the two possibilities, it was decided to re-insert fragments of the 872bp psi sequence back into the J258 construct. However, since the Hind III site had been destroyed in J258, it was first necessary to re-introduce a Hind III site via site-directed mutagenesis (SDM). This was done in the J245 construct to create a new Hind III site between the end of the 5' LTR and the beginning of the psi sequence. The 5' phosphorylated Hind III insertion (#109) and Kpn I deletion (#108) oligos were annealed to the same single strand of template DNA. The oligos were extended and joined to one another by DNA pol I. The product was digested with Kpn I, phenol:chloroform extracted, ethanol precipitated and used to transform E.Coli (strain XL1-blue). Resulting colonies were then screened via Kpn I digestion of their plasmid DNA.

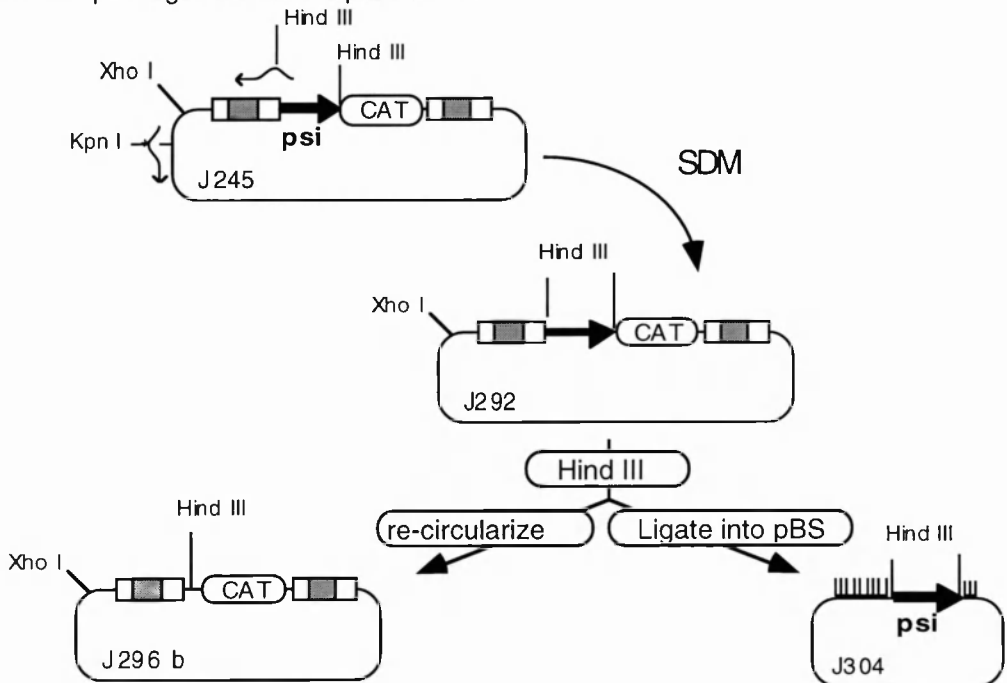


Figure 16. Introduction of a Hind III site to enable removal of the psi sequence as a Hind III fragment. Site-directed mutagenesis (using Stratagene's Chamaeleon[®] kit) was performed to introduce an additional Hind III site into construct J245. The two SDM oligonucleotides are described in the materials section. The 872 bp psi sequence was then removed and cloned into Bluescript for subsequent modifications (J304). Following psi removal, the vector was allowed to re-circularize (via Hind III ligation) to produce construct J296b. This was identical to J258 except for the presence of the new Hind III downstream of the 5' LTR.

Re-insertion of the entire psi sequence in the reverse orientation (construct J296a) led to complete loss of CAT activity, probably due to the presence of complex secondary structures. The possibility that the loss of CAT activity was due to mutations in the CAT gene during SDM was checked by comparing the CAT expression from SDM-derived constructs (J296b & J292) to those which did not undergo SDM (J258 & J245). Results confirmed that the SDM process was not responsible for the loss of CAT expression (fig. 17).

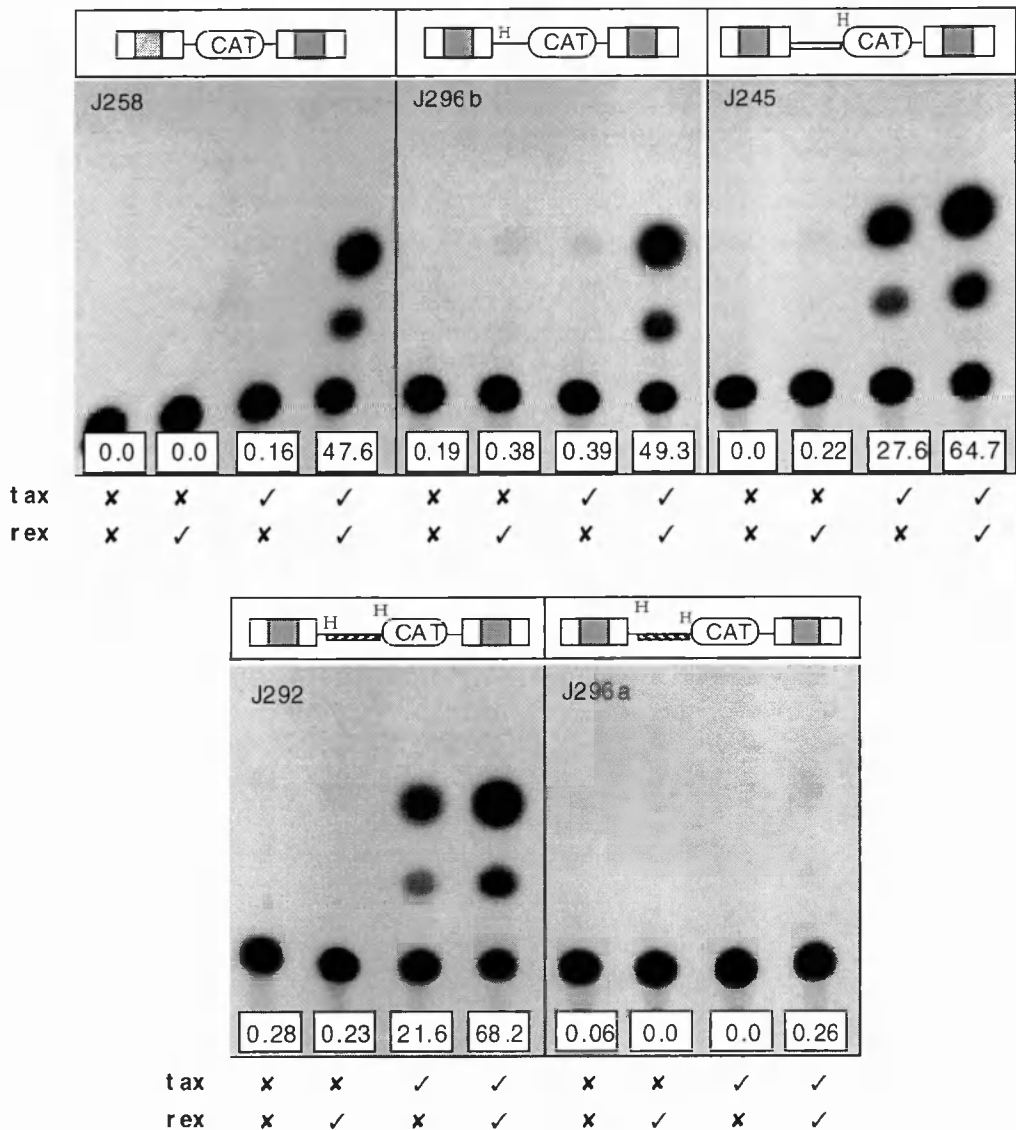


Figure 17. CAT assay to compare expression of SDM-derived and normal constructs. Jurkat cells were transfected with 5µg of each CAT plasmid. 48hrs after transfection, lysates were prepared, standardized for total protein and assayed for their CAT activities. Comparison of the SDM-derived constructs (J296b and J292) to normal constructs (J258 and J245) showed that no damaging mutations had been introduced by SDM and that this was thus not responsible for the zero CAT expression seen in construct J296a. Percentage conversion of chloramphenicol (boxed) is shown .

The 872bp psi sequence, once cloned into the Bluescript II plasmid, contained two unique cloning sites. 201bp inside the 5' end of the psi sequence was an Aat II site and 312bp from the 3' end was a BstE II site. Construct J304 was cleaved using Aat II, Klenow blunted and ligated to Hind III linkers. In parallel, another small amount of construct J304 was cleaved with BstE II, blunted and had Hind III linkers ligated to the blunt ends. Now the 872bp psi fragment could be cleaved by Hind III to yield either 201bp and 671bp fragments or 560bp and 312bp fragments. Each of the four fragments was ligated independently into the Hind III site of construct J296b (fig. 18).

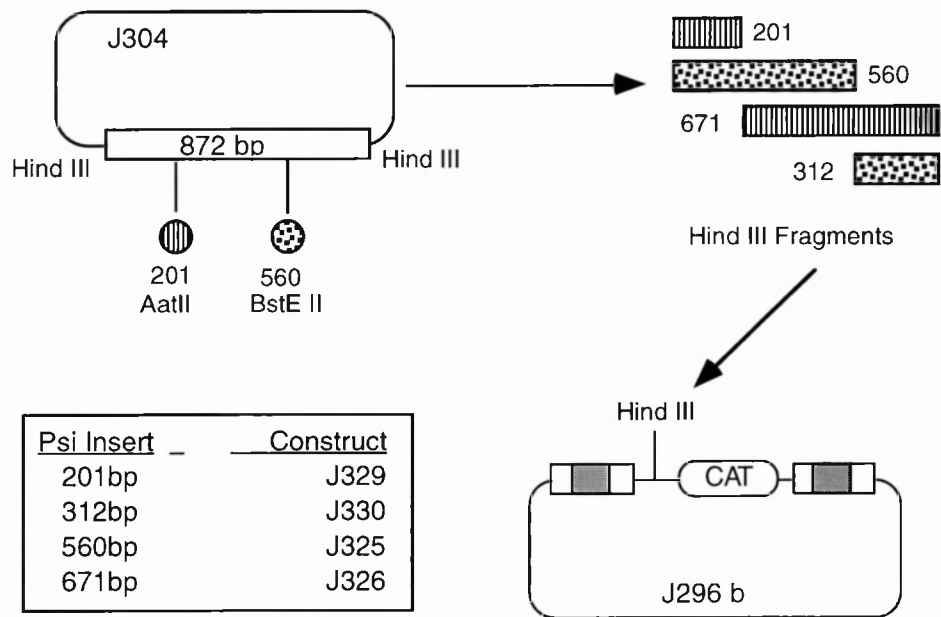


Figure 18. Linker insertion into psi sequence followed by cloning of psi (Hind III) fragments into the SDM construct J296b. The new constructs containing the 201, 312, 560 and 671bp psi fragments in the positive orientations were checked by sequencing and then named J329, J330, J325 and J326, respectively.

These constructs were then used to transfect Jurkat T cells. Lysates were made and assayed for their CAT activities (fig.19).

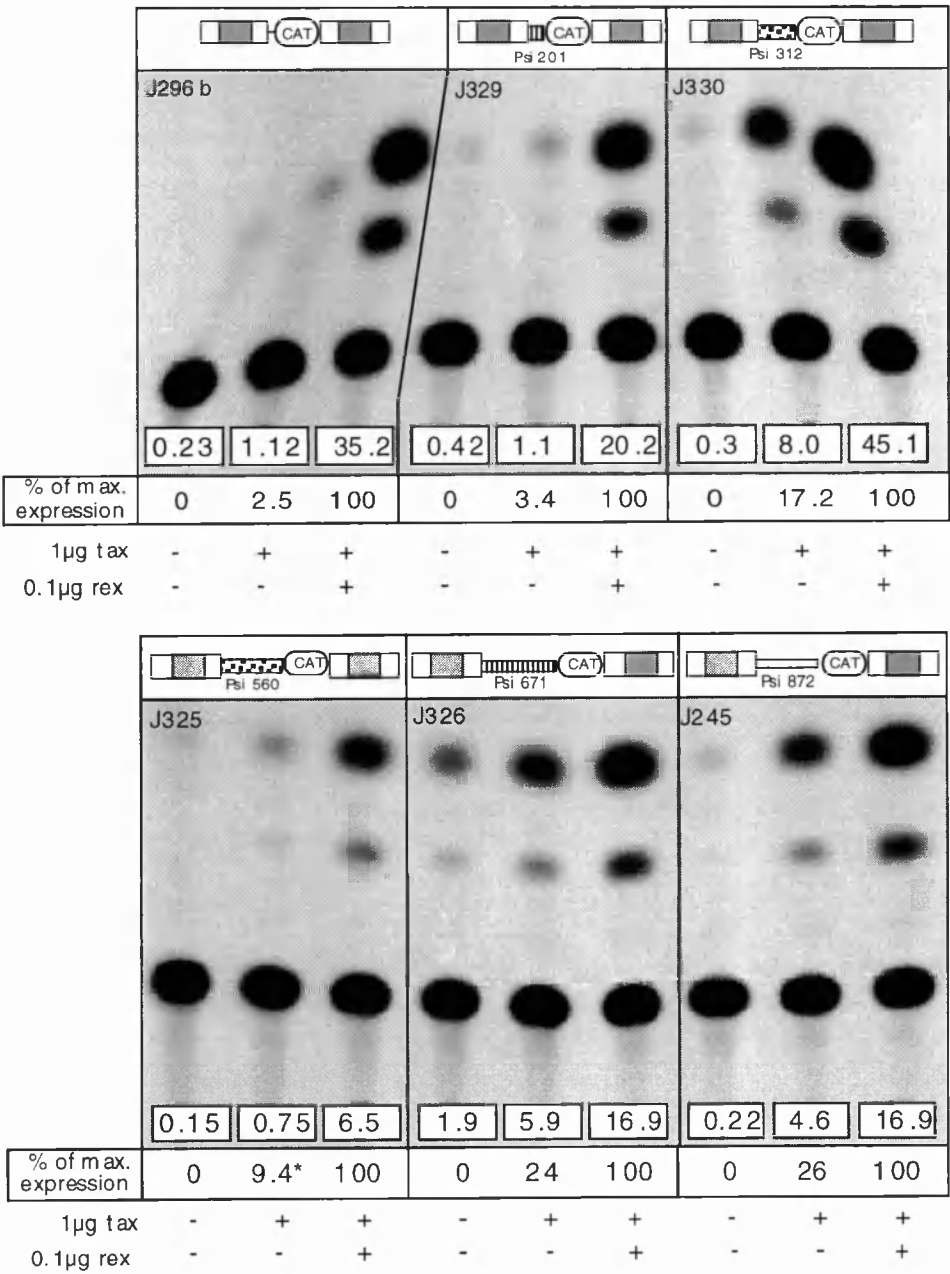


Figure 19. CAT expression assay of psi fragment containing constructs. 5µg of each of the four new constructs was co-transfected with Tax and Rex expressing plasmids. Lysates were prepared, standardized for total protein and assayed for their CAT activities. These were compared to the psi⁻ construct (upper row, lanes 1-3) and to the construct containing the full 872bp psi sequence (lower row, lanes 7-9). % maximum acetylations were calculated for each construct, taking the Rex induced level as the maximum in each case. * The high % of maximum is due to the unusually low figure after rex induction.

From this experiment it was seen that the CTE effect was not size dependent but localized to the 3' end of the psi sequence, within the terminal 312bp. To rule out the possibility that the CTE effect was specific to human T cells, constructs containing psi 312 and psi 560 fragments were used to transfect the COS7 and Hela cell lines (fig. 20).

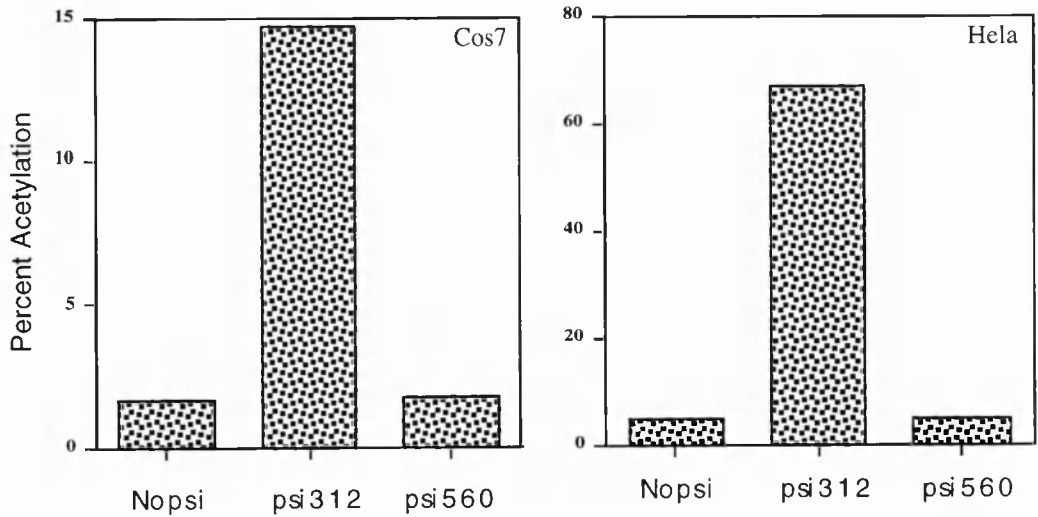


Figure 20. CAT assays repeated in COS7 and HeLa cells. The psi 312 and psi 560 containing constructs were compared to the psi⁻ construct. These transfections were carried out using 15µg of CAT reporter plasmid and 5µg of Tax expressing plasmid. Lysates were prepared 48 hours after transfection, standardized for total protein and assayed for CAT activity.

The pattern was similar in all three cell lines, showing the CTE to be present in the 312 bp fragment of the extended psi sequence. CAT expression levels from COS7 cells were seen to be lower than from HeLa cells. This was due to the high transfection efficiency of the HeLa cells relative to COS7 in this experiment. To further enhance expression levels from COS7 transfected constructs, the SV40 origin of replication (ORI) was inserted into all plasmids of interest. SV40 large T antigen, which is present in COS cells acts to stabilize SV40 ORI containing plasmids in the nucleus as episomes. Expression from episomes yields a larger number of transcripts, which is important when analyzing the size and intracellular location of the RNAs.

• PCR Subcloning of the SV40 Origin of Replication

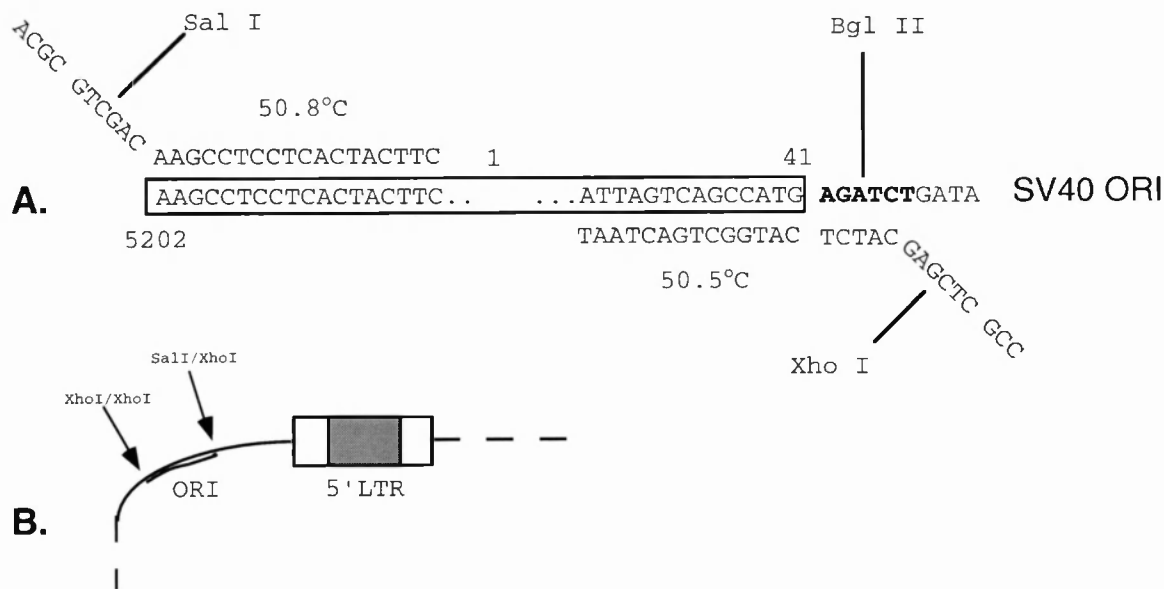


Figure 21. PCR amplification and cloning of the SV40 ORI. Oligos (#116 & 117) were designed to amplify the SV40 ORI sequence from plasmid pL.SEAP (Berger *et al.* 1988) (A). The numbers relate to positions in the SV40 virus. Primers were chosen to anneal at approximately the same temperature and introduce two compatible restriction sites for convenient cloning into the Xho I site, just upstream of the 5' LTR in all HTLV-I based constructs (B). Clones having a single ORI insert in the negative orientation, relative to the HTLV-I promoter, were selected via PCR screening of colonies and verified by DNA sequencing (T7 primer).

COS cells were originally immortalized by the SV40 large T antigen (Gluzman, 1981). The presence of SV40 large T enables cells to maintain SV40 ORI-containing plasmids as episomes in the nucleus. Expression from episomes results in more transcription which is useful when specific RNAs are to be visualized or isolated and purified. The 94bp PCR fragment was inserted into the Xho I site of three constructs which were used to examine the role played by the psi CTE .

Constructs	Before ORI Insertion	After ORI Insertion
Full Length	J258	J337
Full length + psi312	J330	J339
Full length + psi560	J325	J340

Table 1. Change of plasmid nomenclature upon insertion of the SV40 ORI sequence.

Having shown that CAT expression was increased upon introduction of the psi 312 fragment into the full length construct, it was important to determine at which level the regulation was taking place.

The element could have been acting to increase the level of transcription or induce a cryptic splicing event. Splicing involving the 5'LTR splice donor and a cryptic splice acceptor within the psi 312 region would have resulted in splicing out of the CRS element from the 5' LTR. To determine whether either of these two mechanisms was responsible for the psi 312 effect, mRNA was purified from COS7 cells that had been transfected with either the full length (J337) or psi 312 (J339) constructs. The RNA was blotted on to a membrane and probed first with a ^{32}P -labelled CAT probe and then with a glyceraldehyde-6-phosphate dehydrogenase (GAPDH) probe as a control for the amount of cellular RNA present.

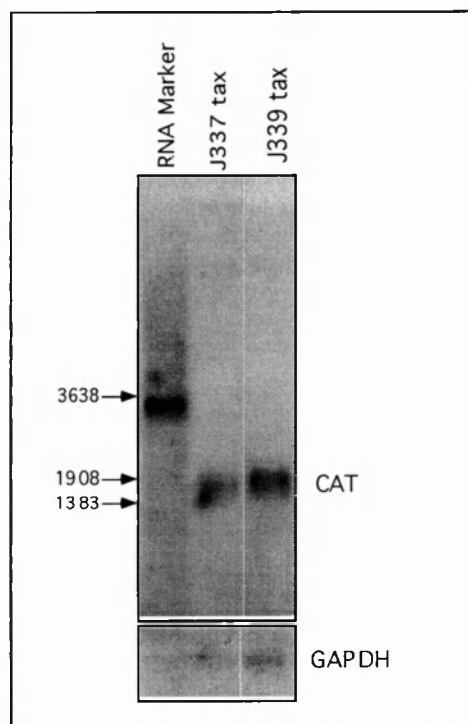


Figure 22. Northern blot of mRNAs obtained after transfection of COS7 cells with constructs J337 and J339. A GAPDH probe was used to allow standardization of the relative amounts of CAT specific mRNAs.

From the Northern blot it was clear that insertion of the psi 312 fragment did not lead to an increase in the steady state level of mRNA. The mRNA band from the psi 312 construct (fig.22 lane 3) was observed at the expected height, indicating that splicing had not occurred.

To test whether the presence of the psi 312 fragment led to transport from the nucleus to the cytoplasm it was important to be able to visualize the specific CAT mRNAs within the transfected COS 7 cells. By using the fluorescence *in situ* hybridization (FISH) technique, it was expected that CAT RNAs would be visible intracellularly.

• Fluorescence In-Situ Hybridization - Determination of CAT mRNA location

Here, a small number of cells (100,000) were seeded on glass microscope slides and Calcium Phosphate transfected. The cells were then fixed and partially permeabilized. Biotin-dUTP labelled probe (single-stranded DNA) was then allowed to enter the fixed cells and hybridize to the RNA molecules. After washing away non-hybridized probe, fluorescein- tagged avidin was then used to detect the RNA-DNA complexes. Cells were examined under the fluorescence or LSM microscope (Zeiss) using a 40x or 63x lens to determine in single cells the localization of specific RNA.

Initially, the FISH experiment was tested on mouse NIH3t3 cells that had been stably transfected with a β -galactosidase expressing construct (RSV-LacZ). Here it was expected that the β -galactosidase RNA would be present in the cytoplasm of all cells.

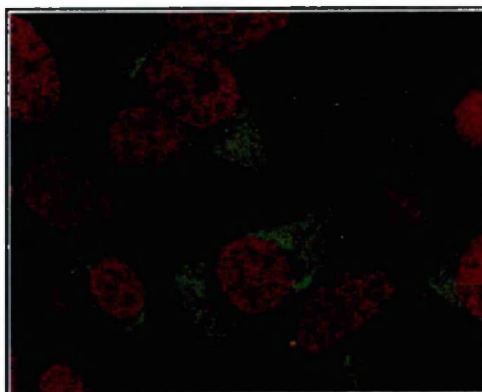


Figure 23. FISH of β -galactosidase expressing NIH3t3 fibroblasts. Here cells were seeded onto glass slides and allowed to become sub-confluent. After being fixed and permeabilized, the cells were probed with biotinylated-dUTP labelled LacZ probe. After washing to remove non-hybridized probe, fluorescein-tagged avidin (green) was added to detect LacZ RNA-DNA hybrids. A short incubation with propidium iodide (PI) was then made to visualize the cell nuclei (red).

Although the FISH worked, it was decided to switch from biotinylated-dUTP to Digoxigenin-11-dUTP as it was observed in non-transfected cells that the small amount of endogenous biotin present was causing a faint cytoplasmic background signal.

Next, to determine whether or not a clear distinction could be made between a nuclear localization and a cytoplasmic localization, the full length (ORI-containing) construct (J337) was used.

It had been shown that upon Tax co-expression alone, a full length construct yielded almost no CAT protein. The additional expression of Rex was required for CAT activity. In this situation, J337 RNAs should be visible in the nucleus in the absence of Rex and in the cytoplasm in the presence of Rex. The full length (J258) construct was used to make a labelled probe via the nick-translation reaction (see methods section). COS7 cells were chosen in combination with plasmids containing the SV40 ORI element.

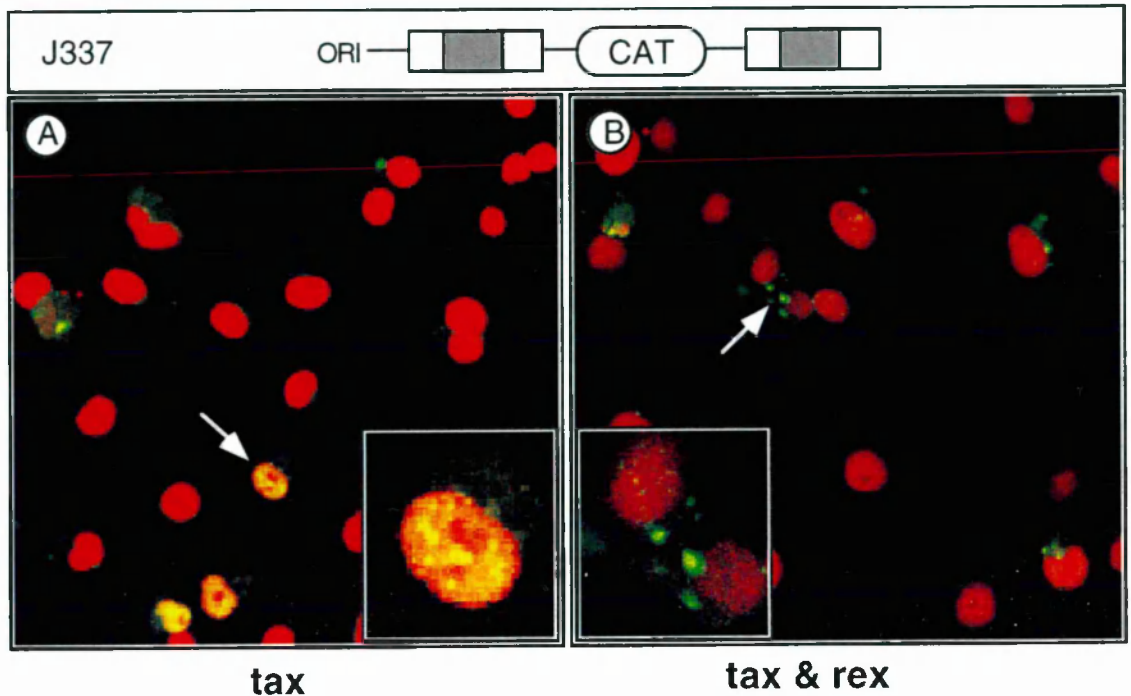


Figure 24. FISH to optimize nuclear/cytoplasmic staining in COS7 cells. Fluorescence *in-situ* hybridization of dUTP-DIG labelled, CAT-specific probe to CAT RNAs inside transfected COS7 cells. Cells were either co-transfected with Tax plasmid (A) or Tax & Rex plasmids (B). Propidium Iodide (PI) was used to stain the nuclei (red). A fluorescein-tagged anti-DIG antibody (green) was used to detect the labelled probe. Where the antibody had a nuclear localization, the product fluorescence was yellow. The dark red structures visible inside the yellow nuclei are the nucleoli.

Here it was possible to clearly distinguish between nuclear and cytoplasmic localizations of CAT RNA.

It was then possible to transfect the psi 312- and psi 560- containing constructs and compare their localization to the full length (psi⁻) construct and the psi 872 containing construct.

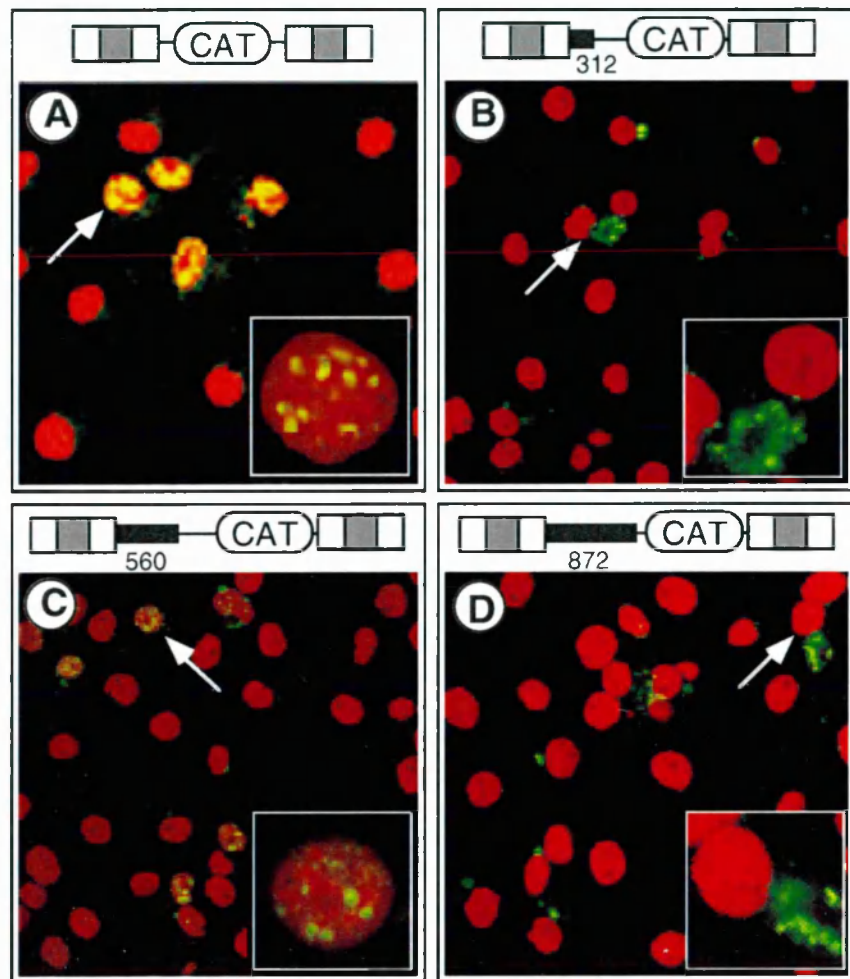


Figure 25. FISH analysis of COS7 cells transfected with psi fragment constructs. Fluorescence *in-situ* hybridization of CAT probe to RNAs present in COS7 cells after transfection with the full length psi⁻ J337 (A), the psi 312- containing J339 (B), the psi 560- containing J340 (C) and the psi 872- containing J245 (D) constructs. Specific CAT RNAs were detected by fluorescein-tagged anti-DIG antibody (green). Cell nuclei were stained by short incubation with PI (red).

It is clear that the psi 312 and psi 872 cis-sequences led to CAT expression by allowing the transcripts to leave the nucleus. The 560bp psi fragment did not allow CAT protein expression, because the transcripts were still mainly trapped in the nucleus. To obtain a statistically sound quantitation of the shift in expression from the nucleus to cytoplasm upon "psi CTE" function, over 200 transfected cells per transfection were counted and scored as having either nuclear or cytoplasmic RNA localizations. The ratio of cytoplasmic localizations to nuclear localizations was calculated and plotted for each construct.

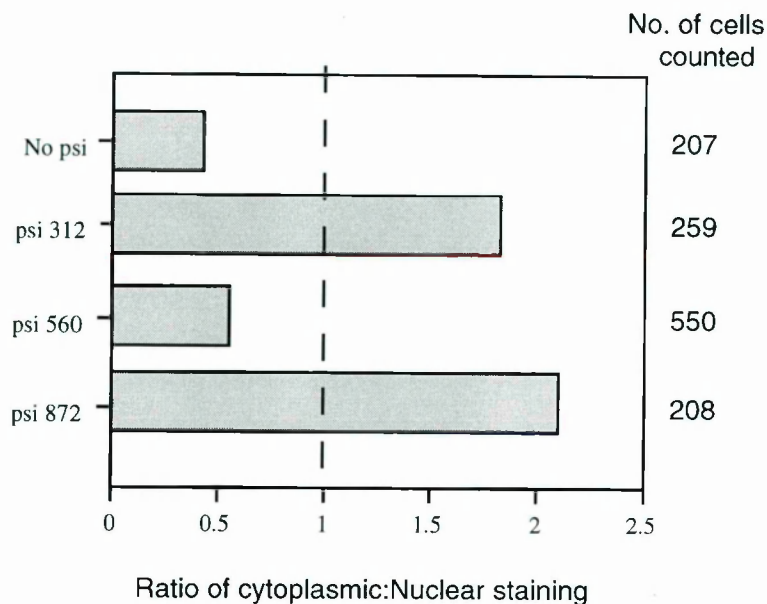


Figure 26. Analysis of the distribution of CAT RNA between nucleus and cytoplasm. This was expressed as a ratio of cells with cytoplasmic to those with a nuclear localization of CAT RNA. Short bars denote nuclear localization - long ones, cytoplasmic. The dotted line marks the equal distribution point (1:1). The total number of transfected cells scored for each transfection is shown in the right hand column.

• Mapping of the psi 312 fragment to the MLV genome

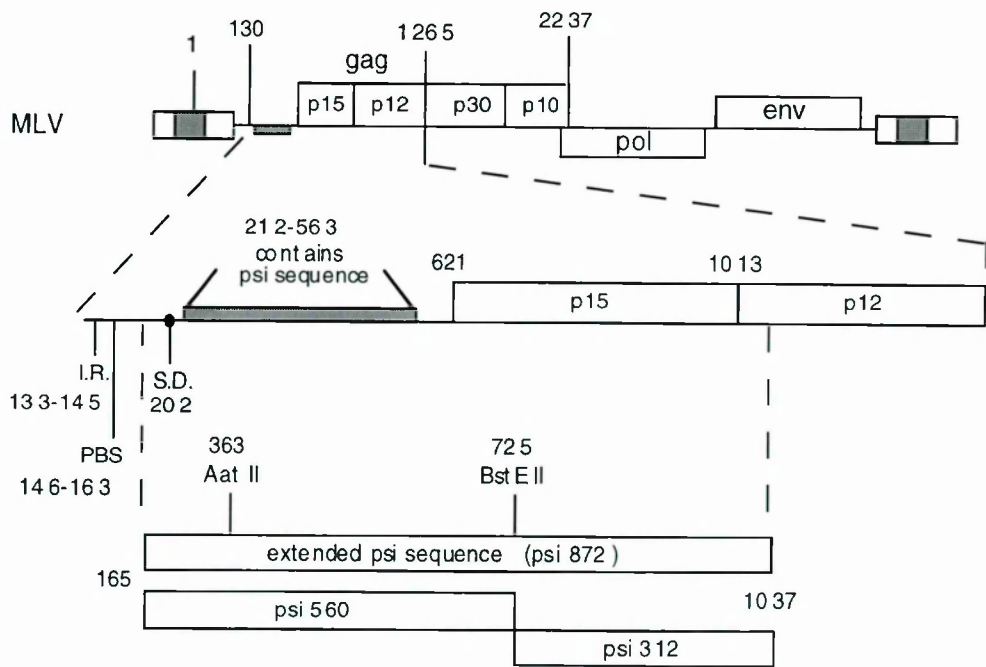


Figure 27. Schematic mapping of the psi 312 fragment to the MLV sequence. Nucleotide (nt) no.1 is capped. MLV genome information was taken from "RNA Tumour Viruses" (Weiss *et al.* 1985). The shaded region from nt. 212-563 is the region required for packaging (psi). The extended psi (psi⁺) allows enhanced packaging, extending to nt. 1037.

Nuclear export of cellular and viral RNA has, in many cases been shown to be mediated by RNA binding proteins which in turn bind nucleoporin-like proteins (Görlich and Mattaj 1996). Competition experiments have shown that although different classes of RNA molecule are bound by specific RNA binding proteins, the array of transport-mediating nucleoporins could be more limited. To determine whether or not the psi312 RNA is bound by a cellular factor, three new plasmids were constructed.

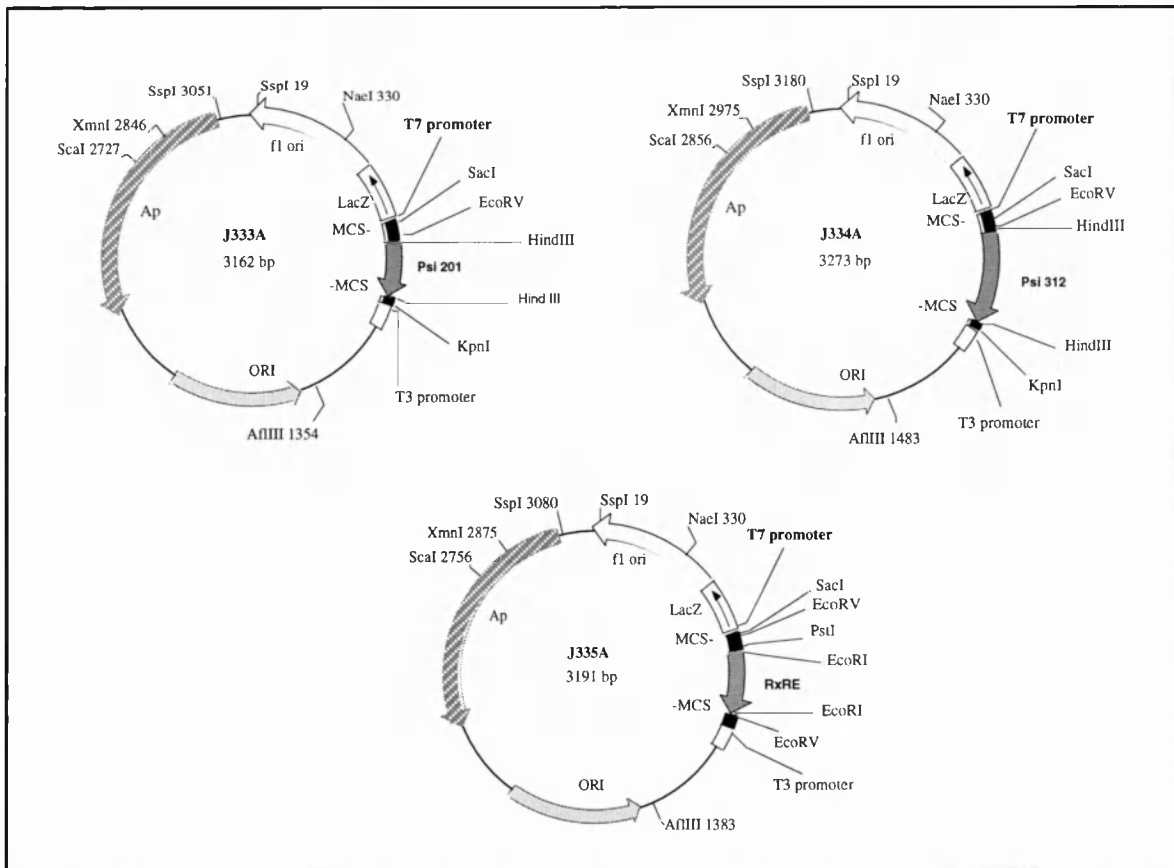


Figure 28. In vitro transcription constructs. The psi201 and psi312 Hind III fragments were ligated into the Bluescript plasmid at the Hind III site (J333A and J334A, respectively). The RxRE was ligated into Bluescript as an EcoRI fragment (J335A). This fragment was PCR amplified as described in SECTION C fig. 36. All three insertions are in the positive orientation with respect to the T7 promoter.

Constructs J333A, J334A and J335A were made to allow the production of radio-labelled RNA transcripts via *in-vitro* transcription from the T7 promoter. J333A was designed to be a negative control as the psi201 fragment did not lead to an increased CAT activity. Construct J335A was designed to be a positive control for binding. In this case, a Rex expressing plasmid would need to be co-transfected.

The labelled RNAs could be used in two different ways to detect a specific binding protein. Firstly, a "gel mobility assay" or "band-shift" experiment could be carried out using nuclear extracts. Binding of a cellular factor would result in a retardation of the labelled RNA on a polyacrylamide gel. In this case RNase inhibitors would need to be used to protect the labelled RNAs from degradation. To avoid problems associated with RNase digestion, North-Western analysis could be carried out. For this the nuclear extracts would be run on a protein gel, blotted onto a membrane and re-natured. This membrane would then be probed using the labelled transcripts. A specific binding protein would be visualized as a clear band.

SECTION C: Identification of a Second CRS

• Presence of a Second Cis Repressive Sequence - inside the 3'LTR

To address this question, an SV40 polyadenylation signal was cloned in place of the 3' LTR in the full length construct (J258). The resulting construct 510 (fig.29) was thus expected to express transcripts lacking the entire downstream non- translated region (NTR).

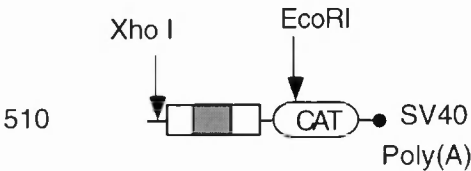


Figure 29: 755.CAT.poly(A) construct, used to look at the importance of the downstream NTR. This was cloned by removing an 850 bp Xho I - EcoRI fragment from construct J258 and cloning it into the Xho I - EcoRI sites of plasmid pBL-CAT 2.

Because transcripts derived from construct 510 would no longer contain a Rex response element (RxRE) it was expected that they would no longer be Rex inducible. As changes made did not effect the Tax response element (TRE) in the 5' LTR, it was expected that 510 would remain Tax inducible. The presence of a negative element inside the 3' LTR would be indicated in a CAT assay if - upon removal of the 3' LTR - CAT expression was seen to become less Rex- dependent (ie. CAT expression in the presence of Tax alone).

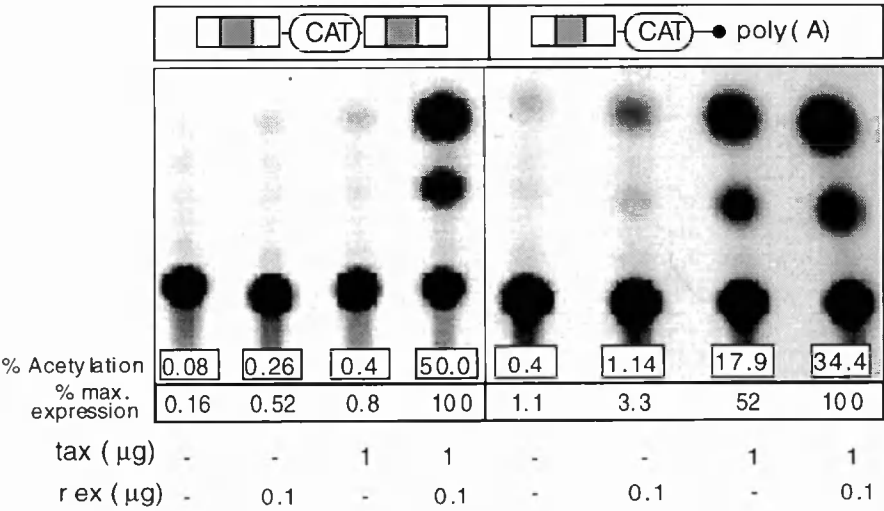


Figure 30: CAT assay to examine the role of the downstream non-translated region. Jurkat T cells were DEAE-dextran transfected with 3 µg of either construct J258 (lanes 1-4) or construct 510 (lanes 5-8). Tax and Rex expressing plasmids were co-transfected as indicated. Lysates were prepared, standardized for total protein and assayed for CAT activity.

Removal of the 3' LTR not only reduced the Rex-inducibility, but also partially removed Rex-dependency of the transcripts produced following Tax expression (compare lanes 3 & 7). This raised the possibility that, as well as a positive regulatory element (RxRE), there could be a Downstream Negative Element (DNE) - with similar function to that of the 5' CRS - present in the 3' LTR.

Although the Rex induction was dramatically reduced, it was not completely removed upon removal of the RxRE-containing 3' LTR. Before the addition of Tax, Rex was able to give a 3- fold increase over the background CAT expression for both J258 and 510 (lanes 2 & 6). The Rex-inductions in the presence of Tax were different. The full length construct (J258) gave a 125- fold increase, whereas, the Rex induction from the 3'LTR-deleted construct (510) is only 2- fold.

Having shown that removal of either the 5' CRS or the 3' LTR led to a partial release of repression, we expected the removal of both elements to result in a larger release, and perhaps to a complete release from repression. To test this, construct J281 was made. It has both the 5'CRS and the 3'LTR deleted.

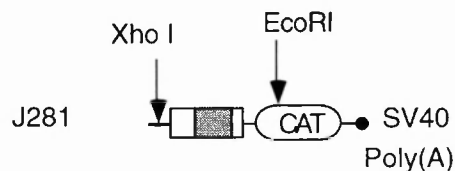


Figure 31. The 628.CAT.poly(A) construct (J281) was constructed by replacing the 850 bp Xho I - EcoRI from construct 755.CAT.poly(A) (510) by that from construct 628.CAT.LTR (J259).

This new construct was then used to transfect Jurkat T cells along with the 3 other parent constructs.

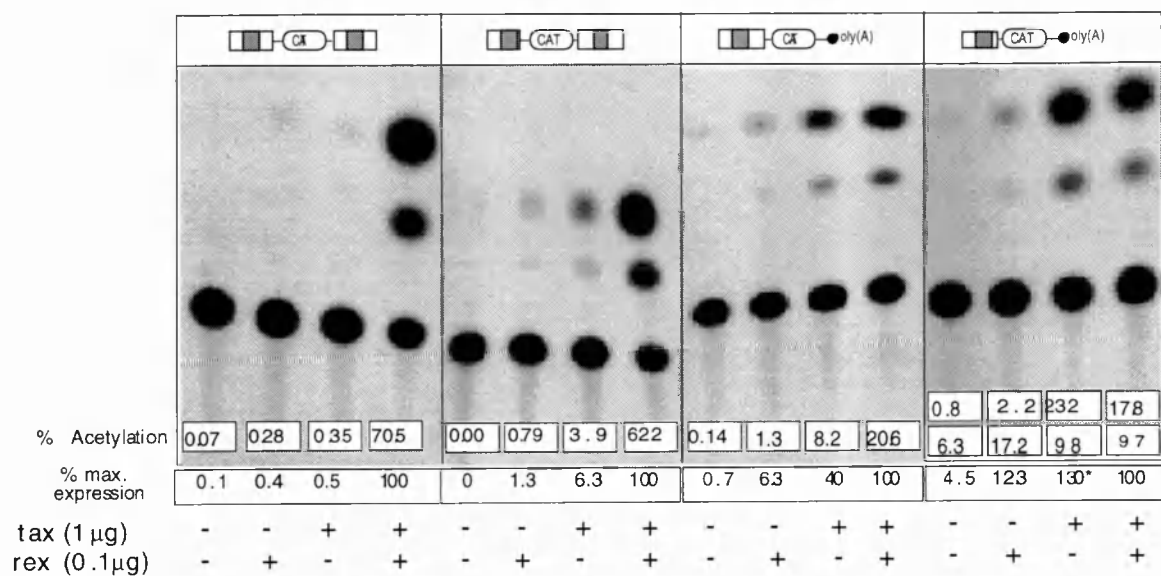


Figure 32. CAT assay showing the result of 3' LTR- and/or 5' CRS- removal. Jurkat cells were transfected with 5µg of each CAT plasmid. Lysates were prepared, standardized for total protein and assayed for their CAT activities. CAT acetylations were so high for J281 (CRS⁻ / 3'LTR⁻) that the CAT assay had to be scaled down (5x less lysate in a 30 minute reaction) and repeated (lanes13-16 upper values). % acetylations and % of maximum expression are shown for each reaction. % maximum acetylation is calculated relative to the rex induced state. *The slight reduction observed in the repression released construct upon Rex expression is due to promoter squelching.

Removal of both the CRS and the 3' LTR did indeed lead to a complete removal of repression. The 2-3 fold rex induction seen in the 3' LTR construct (fig 32. lanes 9-12) is no longer seen when the CRS is deleted (fig 32. lanes 13-16) suggesting that either the putative Rex-binding motif within the 5' LTR has been destroyed or that upon removal of both the CRS and the 3' LTR there are no repressive sequences remaining and the transcripts are free to be expressed Rex-independently.

• **Narrowing the search to the R region of the 3'LTR**

Brighty and Rosenberg (1994) reported that the HIV Rev response element (RRE) contains sequences which confer Rev dependency. Removal of the RRE was shown to lead, not only to the removal of Rev inducibility but also to the removal of Rev dependence, meaning that transcripts were able to leave the nucleus without first being bound by Rev. The HIV RRE, in contrast to the RxRE of HTLV-I, is present inside the coding region of the HIV *env* gene. Prompted by these findings for the HIV RRE, a deletion was made, which specifically removed the RxRE from the 3'LTR.

• Specific Removal of the RxRE from the 3'LTR

Two pairs of primers (#93 & 105 and #106 & 107) were used to PCR amplify the U3 region and U5 regions separately (see materials section for oligo sequences). The PCR products were cut with restriction enzymes and cloned back into the source plasmid via a 3 - fragment ligation, in place of the 3' LTR. Care was taken to leave both the poly A signal and poly A site (at either end of the RxRE) intact and also to maintain the same "physical" distance between the two elements. The sequence between the two elements was, however, mutated to introduce an Xba I site in preparation for the possible re-introduction of partial RxRE sequences, later on. The resulting plasmid was tested using restriction enzymes. It was found that the introduced Xba I site contained a dam methylated sequence and could, therefore, not be cleaved by Xba I. This problem could be partly overcome by transfecting either the DNA or ligation mix into a dam methylase free strain (*E. Coli* strain JM101).

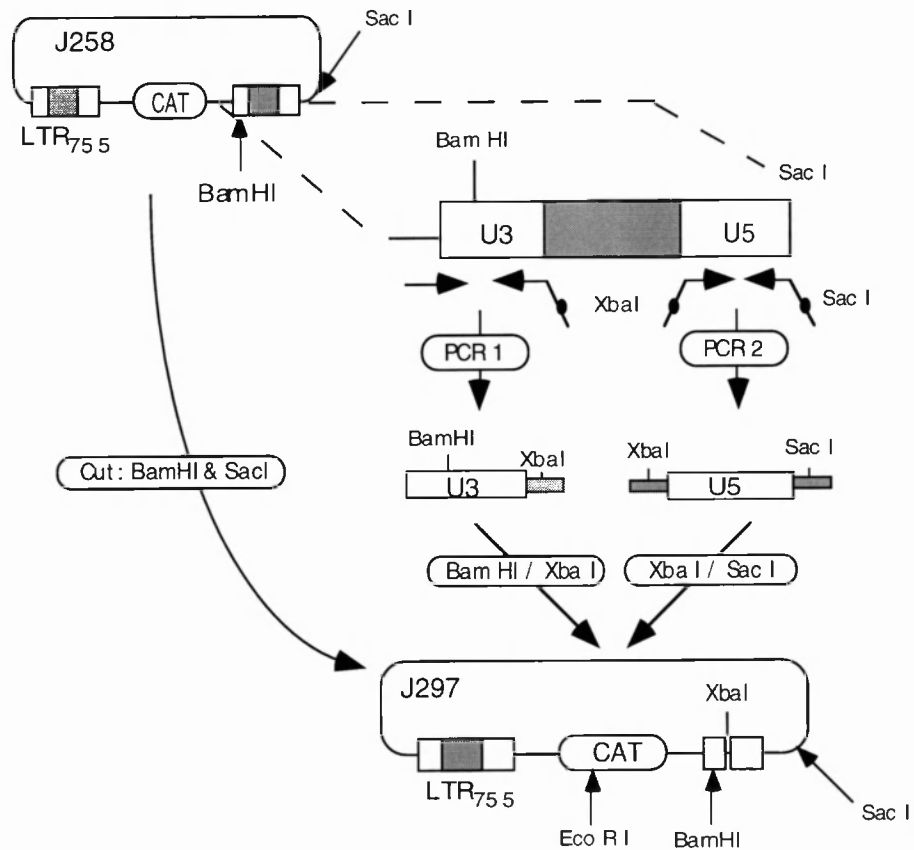


Figure 33. Specific removal of the RxRE via 2 PCRs and a 3-fragment ligation. Construct J258 was cut with Bam HI and Sac I to remove the 3' LTR. The two PCR products (U3 and U5) were cut with enzymes Bam HI & Xba I and Xba I & Sac I, respectively before being ligated with the large J258 fragment overnight at 16°C. Mini-prep DNA was sequenced to ensure that no PCR mis-incorporations had been made.

The resulting construct 755.CAT.U3 / U5 (J297) was cut with restriction enzymes EcoRI and Sac I to remove most of the CAT gene and all of the new 3'NTR. This fragment was then cloned into the LTR628.CAT.LTR in place of the 3' LTR (EcoRI - Sac I) to create a construct with the CRS as well as the RxRE deleted (construct J294). These two constructs were then transfected into Jurkat cells, which were then processed for CAT assays.

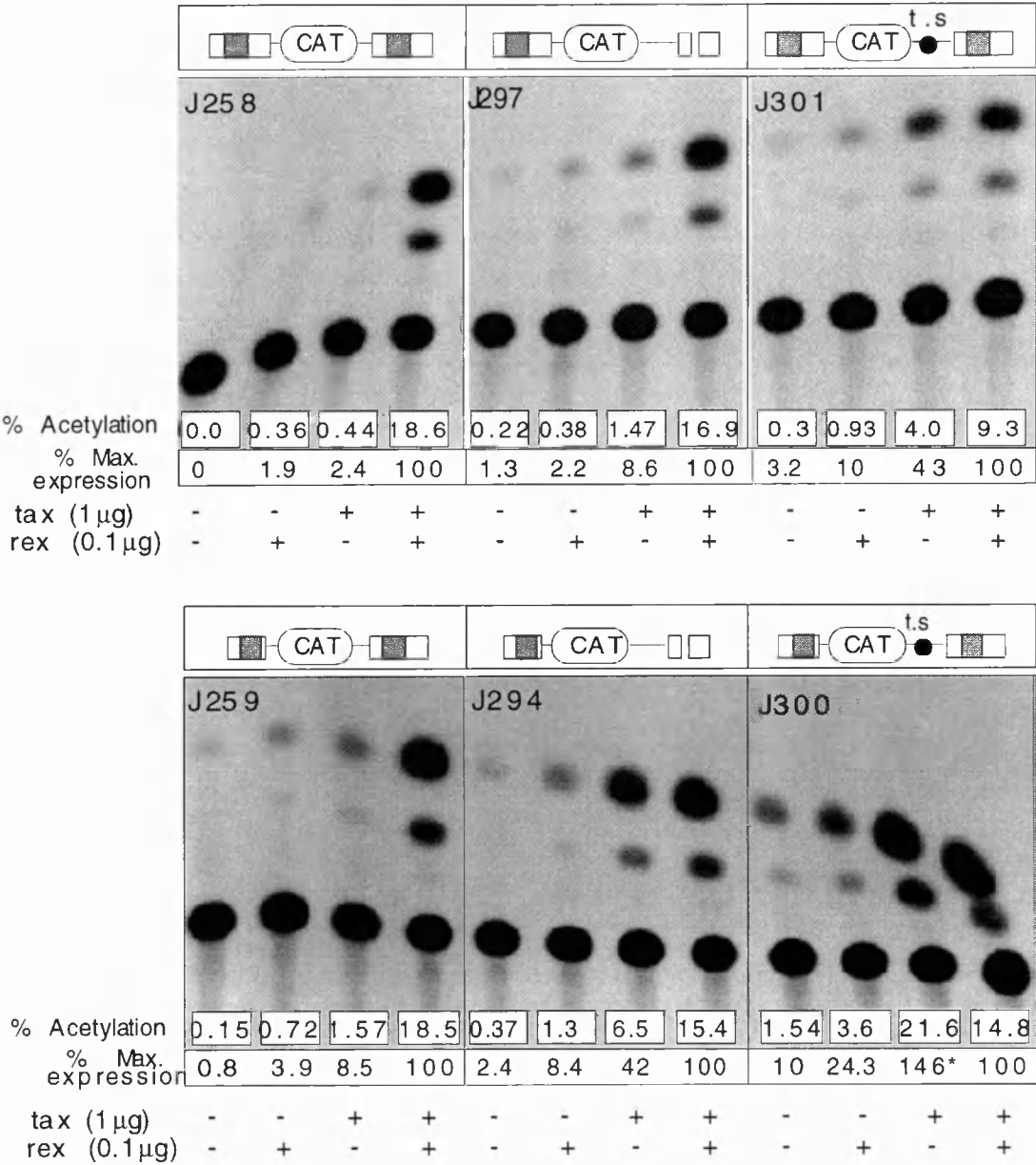


Figure 34: Effect of RxRE removal upon Rex dependency and Rex inducibility. Jurkat cells were transfected with 5µg of each CAT plasmid. Lysates were standardized for total protein and assayed for CAT activity. Constructs J301 and J300 were made by cloning a CAT-poly(A) Hind III fragment from the plasmid pBL-CAT2 in place of the normal CAT cDNA (Hind III - Bgl II). These were used as controls, similar to constructs which lack the 3'LTR completely (510 and J281 respectively).* As fig. 35.

This experiment was repeated using the same CAT constructs and Jurkat cells but this time using the 510 and J281 constructs as controls.

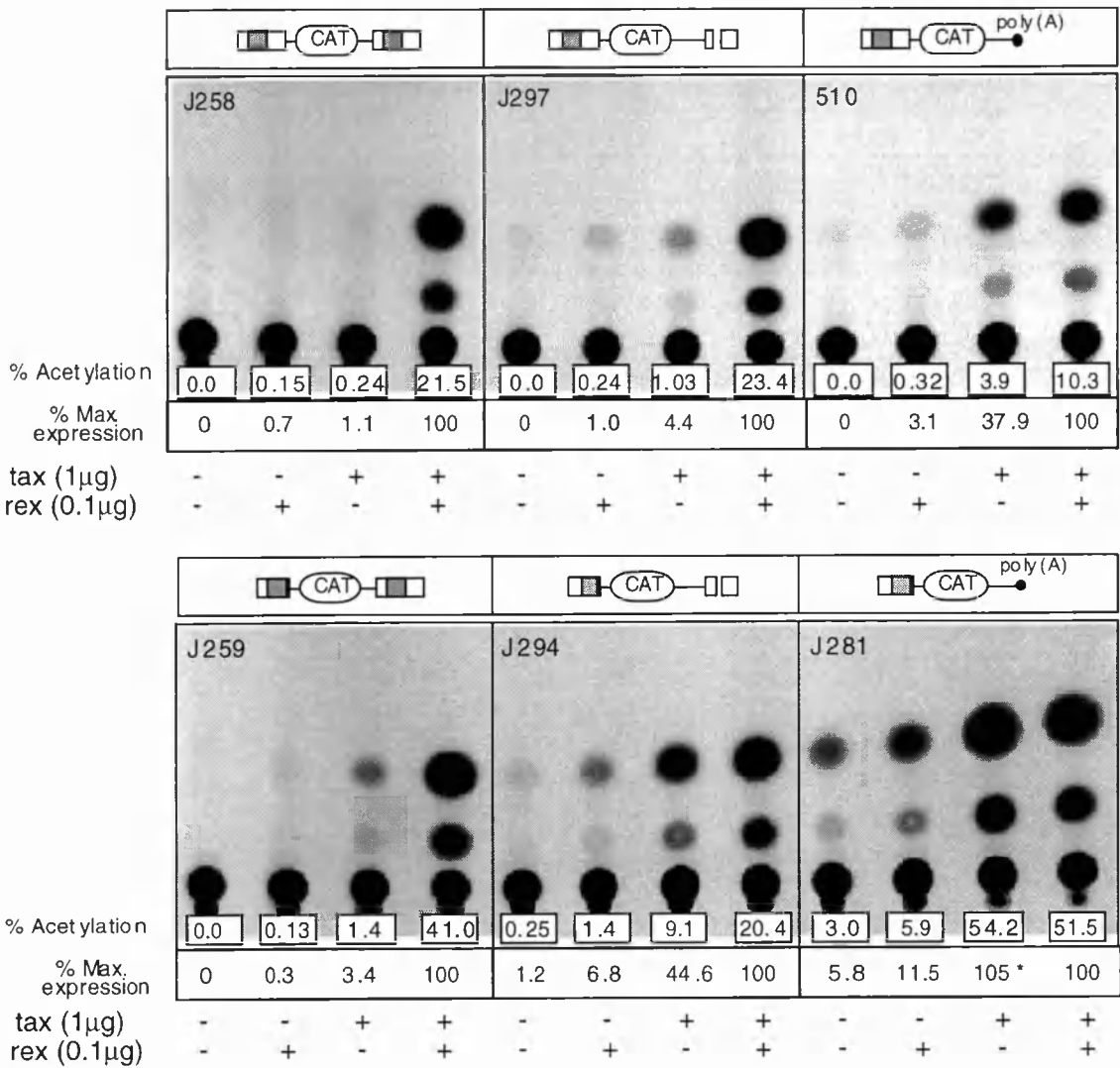


Figure 35. Effect of RxRE removal upon Rex dependency and Rex inducibility. Repeat of previous experiment using 3'LTR-deleted plasmids as controls (510 & J281). * This value is above 100% because the squelching effect observed upon addition of Rex and Tax expression plasmids.

From these experiments, it was clear that removal of the RxRE led to a removal of Rex dependency, indicating that the presence of the RxRE indeed had a repressive influence upon CAT expression. Interestingly, the increase in CAT activity upon removal of the RxRE was similar to that seen following the removal of the CRS. Also, removal of both the CRS and RxRE led in both cases to a more than cumulative increase in CAT expression. However, RxRE removal did not result in the same level of release as did removal of the complete 3' LTR (see controls J301 & J300 and 510 & J281), hinting that the negative element present in the 3' LTR could extend upstream, into the U3 region.

• Specific Insertion of the RxRE

If a DNE is present as part of the RxRE, then it should also function outside the context of the HTLV-I LTR. In work done by Seiki *et al.* (1988) in which the positive regulation, mediated by the RxRE was described, it was noticed that RxRE introduction led to a reduction in basal CAT expression. However, this observation seems to have been overlooked. To examine this more carefully, the RxRE was PCR cloned using oligos #113 and 114, and inserted just upstream of the SV40 poly A signals in constructs 510 and J281.

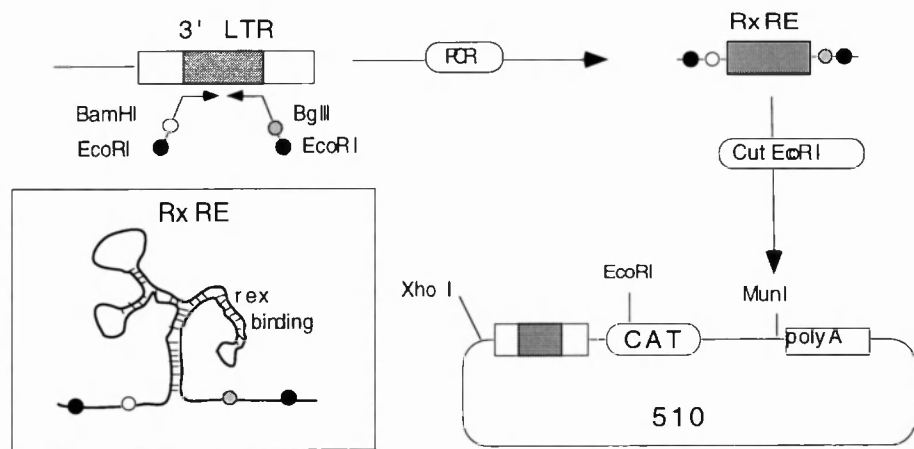


Figure 36: PCR cloning and insertion of the RxRE. This was inserted just upstream of the SV40 poly A signal in construct 510. The PCR product was cut with EcoR I and inserted into the Mun I site next to the poly A signal to give construct J320A. The same PCR fragment was also cloned into construct J281 to give the CRS⁻ variant (J331).

Constructs J320A and J331 were obtained following insertion of the RxRE into constructs 510 and J281, respectively. As the RxRE element insertion was not a directed cloning, both orientations were obtained. Constructs containing the negative orientation (J320B & J332) were also examined for their influence upon CAT expression. These were then used along with the parental constructs to transfect Jurkat T cells. Lysates were made and assayed for their CAT activities.

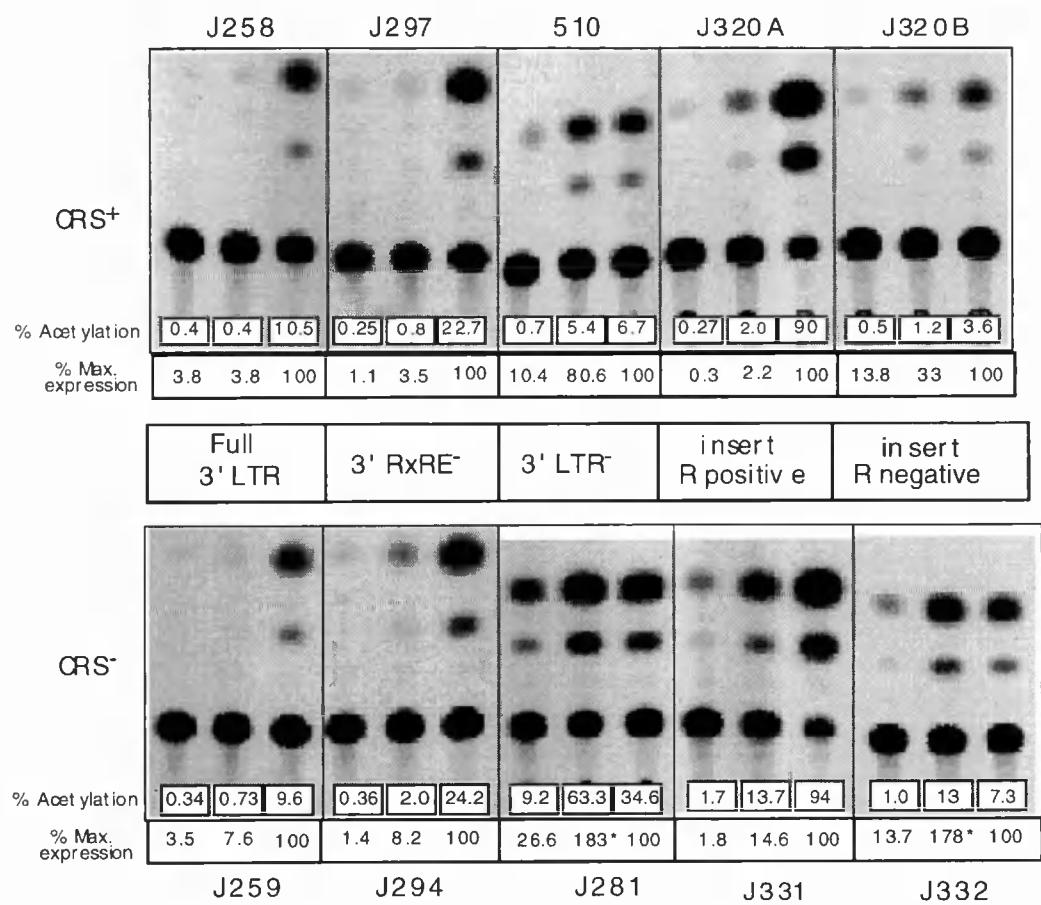


Figure 37. CAT assay to compare the different RxRE deletions and insertions. Constructs in the upper panel all contain the CRS. Constructs in the lower panel have the CRS deleted. Removal of the RxRE (J297 & J294) or 3'LTR (510 & J281) was compared to the constructs containing a full 3'LTR (J258 & J259). Insertion of the RxRE into the 3'LTR- constructs in either the positive orientation (J320A & J331) or the negative orientation (J320B & J332) was compared to the 3'LTR- constructs (510 & J281). Percent acetylation values and % maximum expression values (max. acetylation of 100% with Tax and Rex) are shown for each reaction. * High value is partly due to squelching.

Insertion of the RxRE in either the positive or negative orientation led to a 3-4 fold reduction in CAT expression. This experiment was repeated in COS7 and Hela cells.

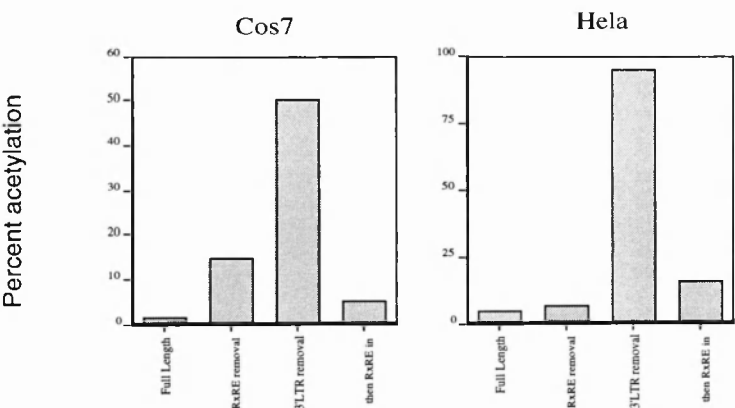


Figure 38. Result of RxRE insertion / Deletion on CAT activities in COS7 and HeLa cell lines. Cell lines were transfected with the full length construct (lane 1), the RxRE-deleted construct (lane 2), the 3'LTR-deleted construct (lane 3) and the 3'LTR-deleted construct having the RxRE re-introduced (lane 4). A Tax expressing plasmid was co-transfected in each case.

The same pattern was seen in all three cell types. Of the two adherent cell types, the clearest differences were seen in the COS7 cells. Five constructs were modified via insertion of the SV40 ORI element just upstream of the 5'LTR (see section B for cloning details).

The 94bp PCR fragment was inserted into the Xho I site of five constructs which were used to examine the role played by the 3' RxRE on expression regulation .

Constructs	Before ORI Insertion	After ORI Insertion
Full Length	J258	J337
CRS ⁻	J259	J338
3' RxRE ⁻	J297	J341
3' LTR ⁻	510	J342
CRS ⁻ /3'LTR ⁻	J281	J343

Table 2. Change of plasmid nomenclature upon insertion of the SV40 ORI sequence.

It has been shown in CAT assays that the full length construct (J258) was highly Rex-dependent and that this dependence could be partially or completely removed by the various deletion / insertion mutations (see figures 34, 35 and 36). It was decided to examine the level of this regulation.

It has been suggested that the presence of the 5' CRS traps transcripts in the nucleus and that binding of the HTLV- I Rex protein to the 3' end of transcripts overcomes the block, allowing transcripts to enter the cytoplasm (Seiki *et al.* 1990). We wanted to determine whether removal of the above mentioned sequences of interest would indeed lead to a shift in CAT RNA distribution between the nucleus and cytoplasm, or whether they were acting via other mechanisms. It was decided to analyze the location of CAT RNA via Fluorescence *In-Situ* Hybridization.

• Fluorescence *In-situ* Hybridization (FISH)

It was shown in section B, that the FISH protocol worked (fig. 21) and that a clear difference could be seen between nuclear and cytoplasmic localizations of specific mRNAs (fig. 22). The background staining of the anti-digoxinin antibody was seen to be very low indeed in the non-transfected COS 7 cell control (fig.39A).

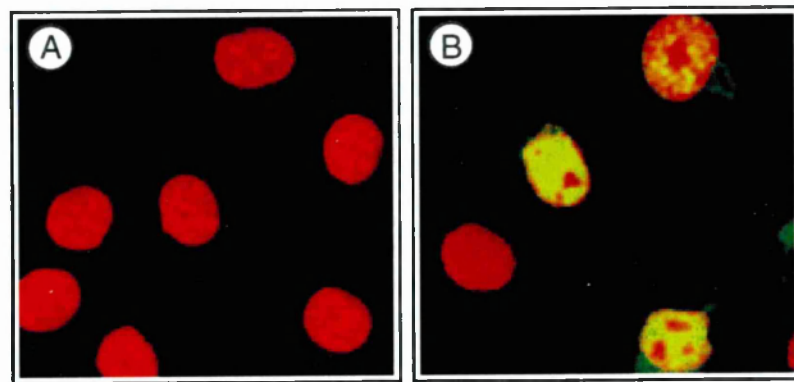


Figure 39. FISH of non-transfected COS cells (A) and COS transfected with the full length (J337), ORI-containing construct (B). Here the fixed cells were permeablized and probed with digoxigenin-11- dUTP which was detected by a fluorescein-tagged anti-DIG antibody (green). Propidium iodide was used to stain the nuclei (red). The fluorescent product of green and red is yellow.

Transfection conditions were optimized for the full length construct (J337) and the CRS⁻/3'LTR⁻ construct (J343) in COS7 cells on glass microscope slides. Three different amounts of CAT reporter were used (5, 10 and 15 µg). Cells were harvested either 2, 3 or 5 days after transfection (To prevent cells becoming too dense, different cell numbers were plated out the day prior to transfection). Optimal conditions were found to be: 15 µg CAT plasmid, 5 µg Tax expressing plasmid and 1µg Rex expressing plasmid (when required). The standard BES transfection protocol (see methods section) was applied and the cells harvested 3 days post-transfection. It was seen to be very important to use cells that had not been in culture for too long (more than 2 months after thawing).

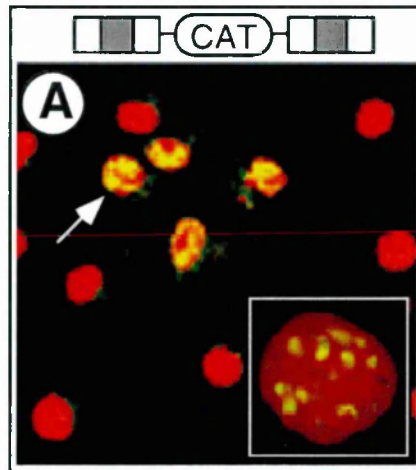


Figure 40A. FISH of COS 7 cells transfected with the full length (J337) construct and the Tax expressing plasmid. Cells were harvested 3 days after transfection. The marked cell (white arrow) magnification (boxed) was made at a lower fluorescein sensitivity to enable more specific intra-nuclear visualization.

Constructs containing the various combinations of CRS and DNE deletions and insertions were then used to transfect COS 7 cells and examined using FISH to determine the function of the various elements with respect to the preferential localization of CAT RNA.

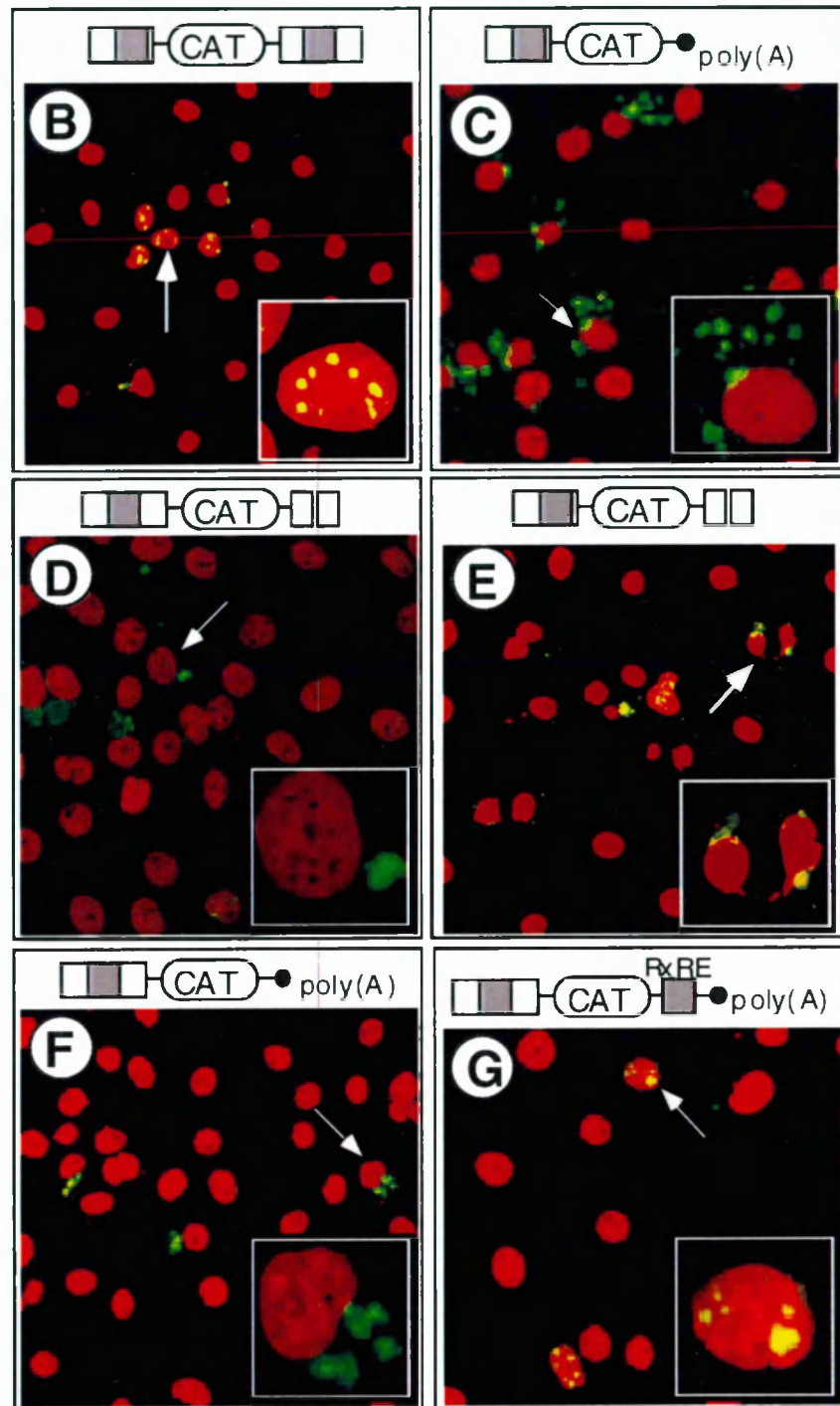


Figure 40B-G. FISH of COS7 cells after transfection with 15 μ g of constructs J338 (B), J343 (C), J341 (D), J294 (E), J342 (F) and construct J320A (G). All transfections included 5 μ g Tax expressing plasmid. Cells were harvested three days after transfection then fixed, permeabilized and probed with DIG-labelled dUTP probe. This was then detected by fluorescein- tagged anti-DIG antibody (green). PI was used to stain nuclei red.

The degree of cytoplasmic or nuclear staining for each transfection was quantitated. For this more than 200 transfected cells per transfection were counted and scored for exhibiting either nuclear or cytoplasmic distribution of CAT mRNA. The results for each transfection were then expressed as a ratio of nuclear to cytoplasmic staining and plotted together to allow comparison (fig. 41).

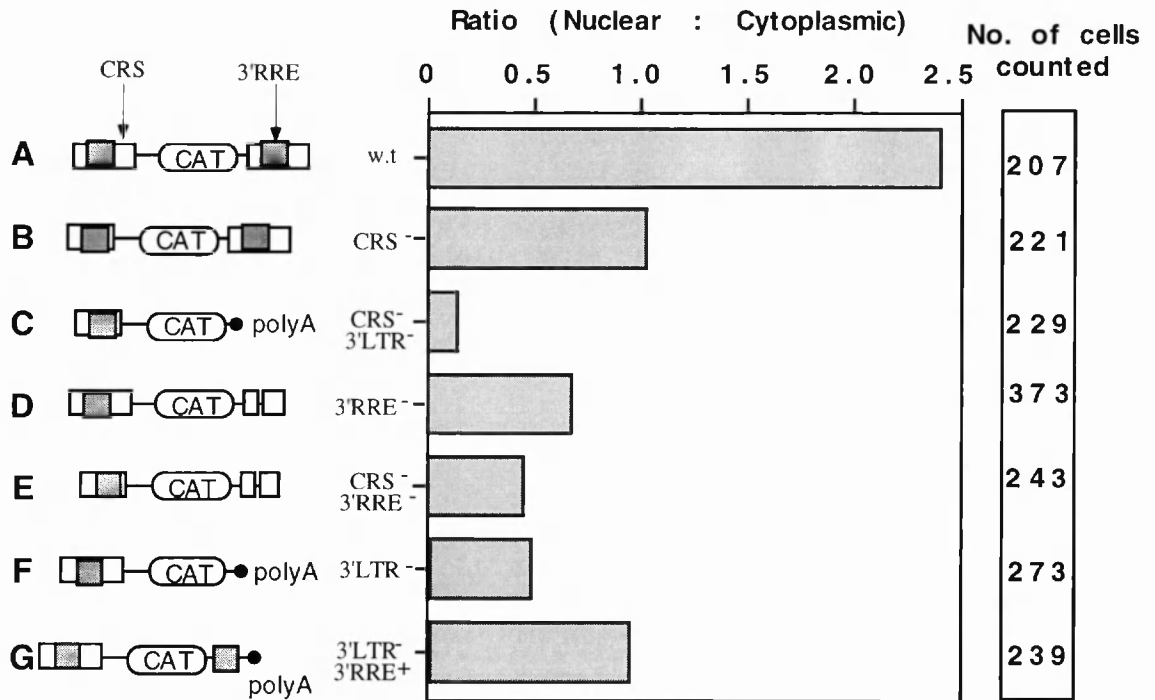
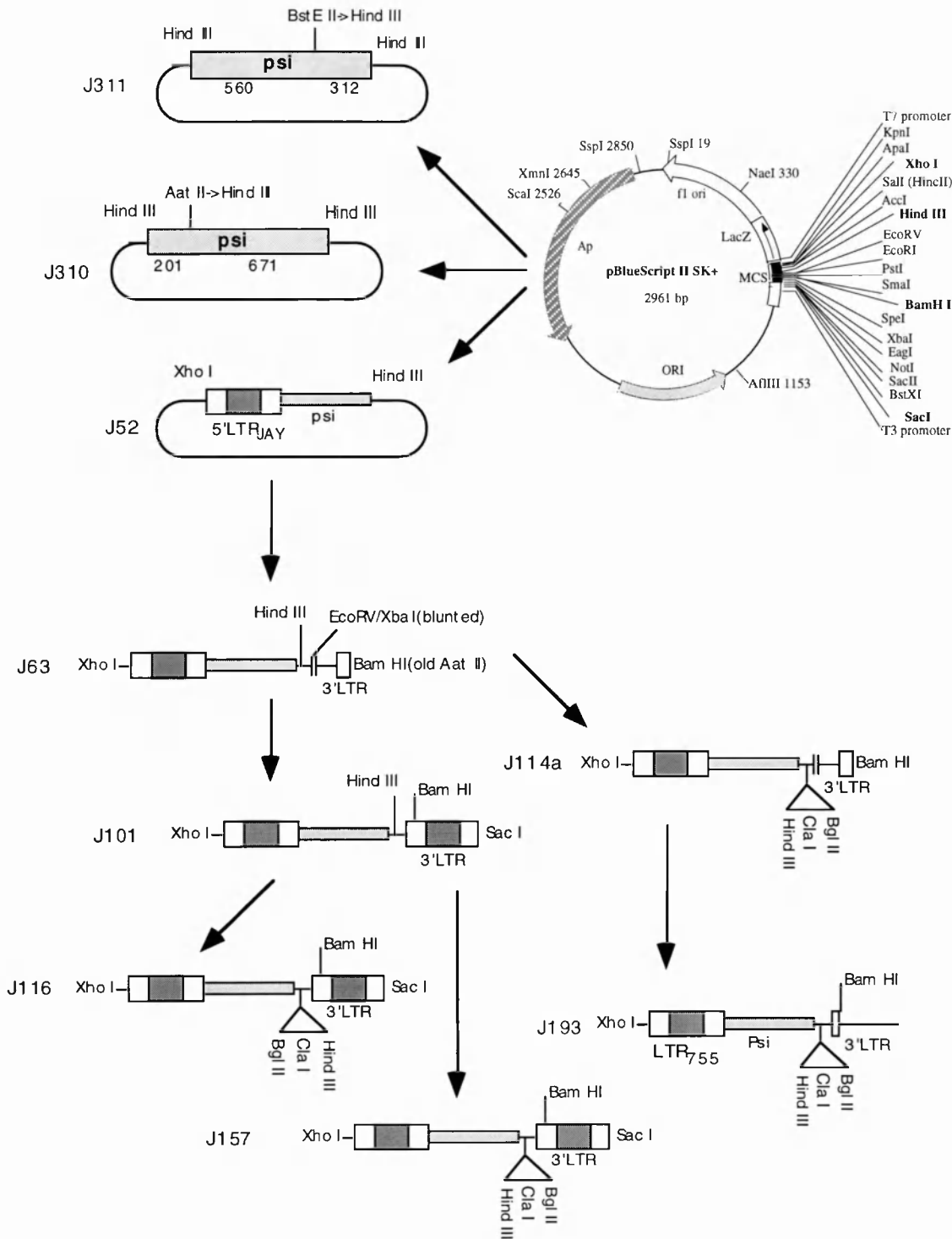
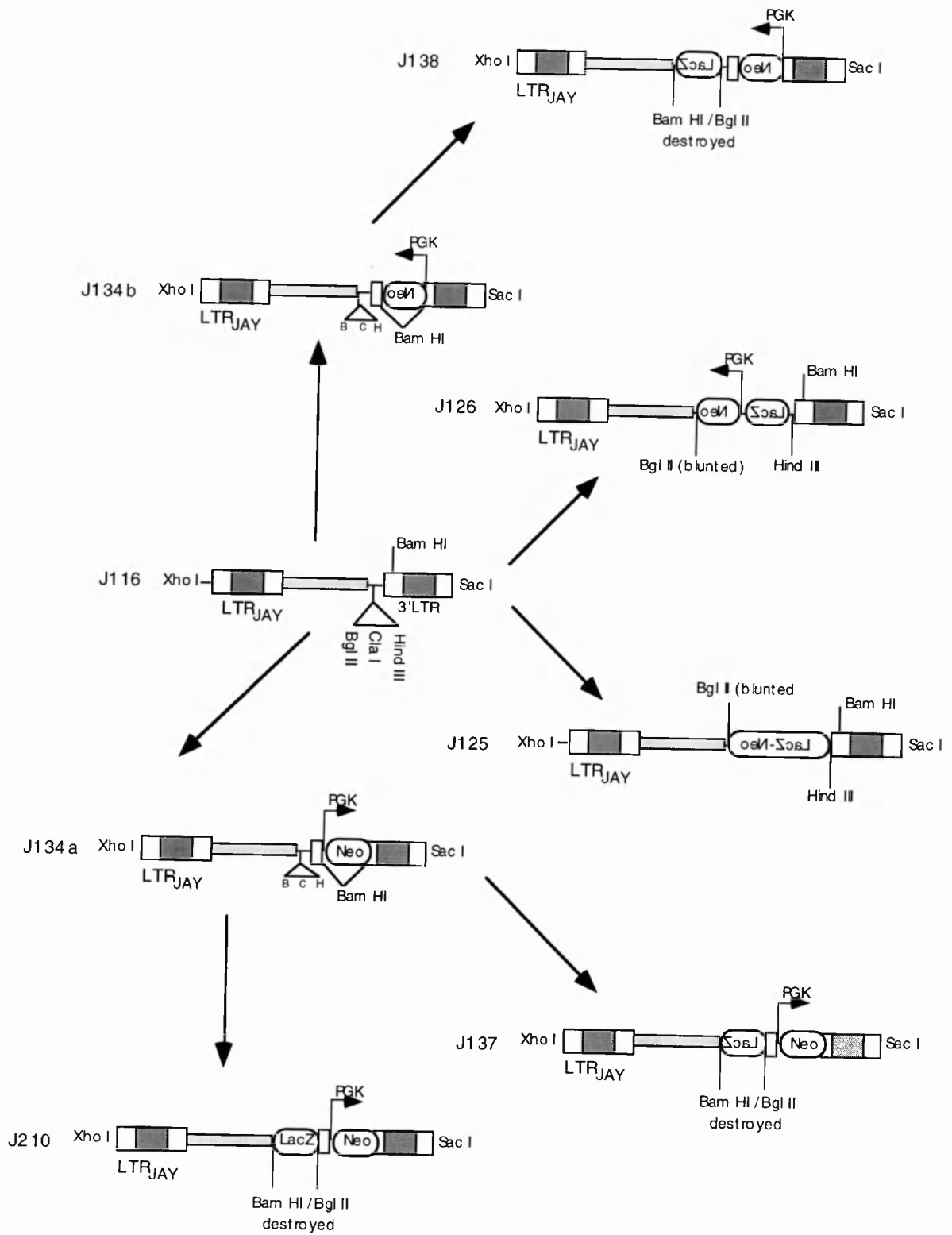
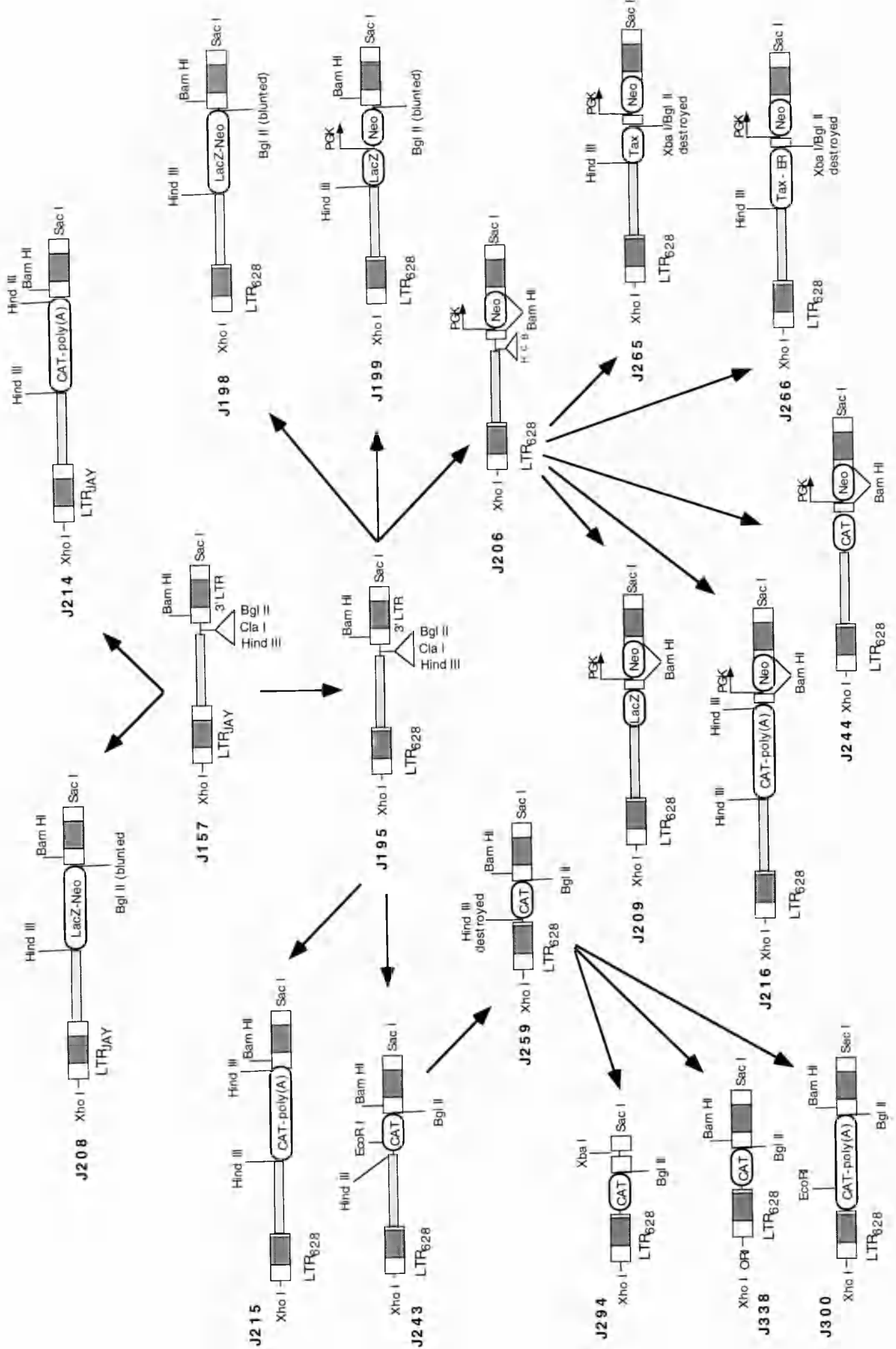


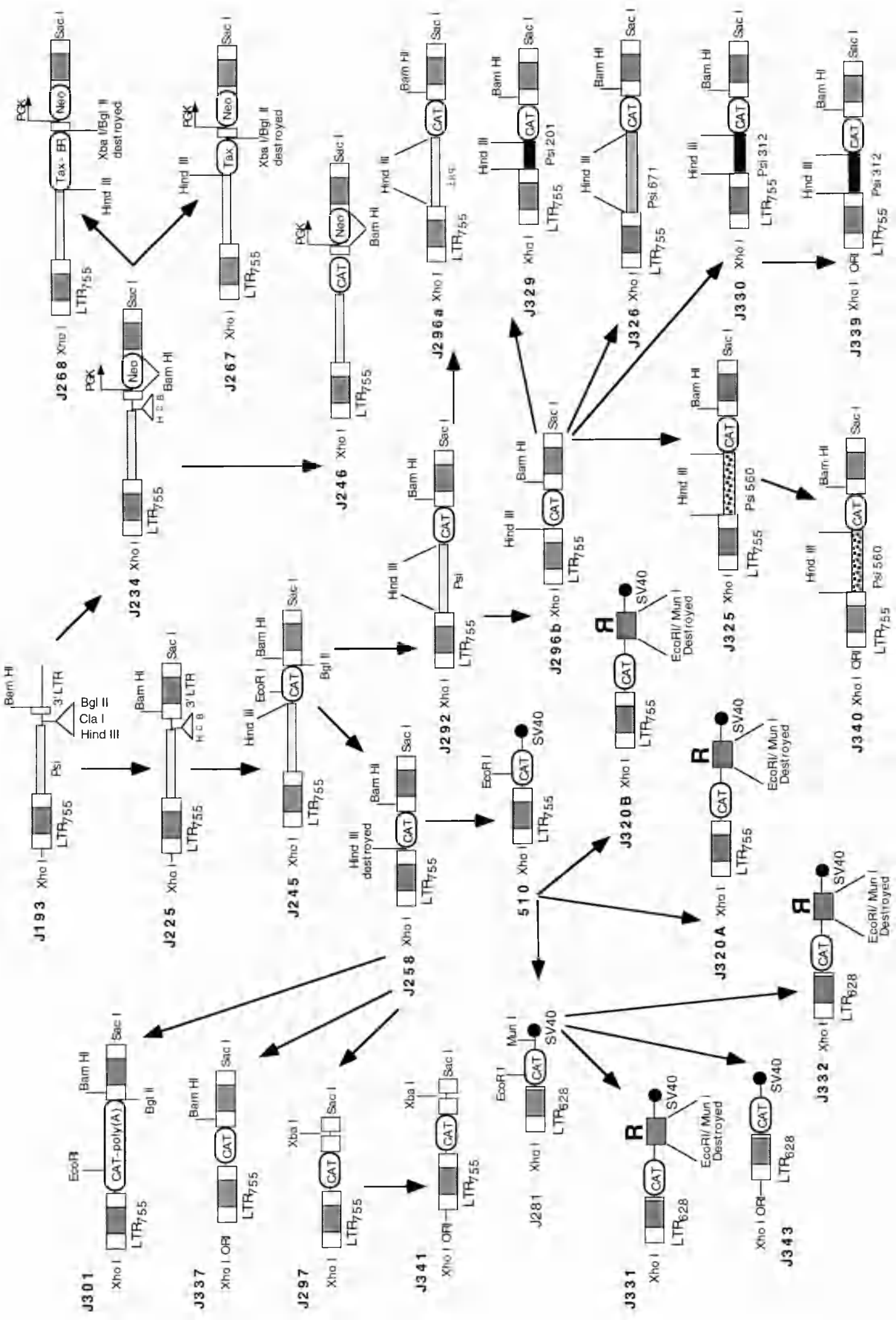
Figure 41. Quantitation of nuclear : cytoplasmic staining ratios. Longer bars indicate more nuclear staining. The full length construct (A) showed very high nuclear staining, RNA being trapped in the nucleus. Removal of the 5'CRS (B) led to a clear reduction in repression. Removal of both the CRS and the 3' LTR (C) led to a complete release, indicated by almost complete cytoplasmic staining. Removal of the 3' RxRE alone (D) led to an even larger release than removal of the CRS. An additional effect was seen upon removal of both the CRS and the RxRE (E). Removal of the 3' LTR (F) led to an even larger release than either removal of the CRS or RxRE, again hinting that the negative element could extend into the U3 region. Re-introduction of the RxRE into the 3'LTR- construct led to a shift back in the direction of nuclear localization, indicating that the level of repression had increased.

SECTION D: Sub-cloning Overview (Flow diagrams)









Rex regulation of HTLV-I is mediated via positive and negative elements in both long terminal repeats

A downstream negative element (DNE) within the 3' LTR

Rex has been shown to function posttranscriptionally in promoting the expression of HTLV-I unspliced and partially spliced transcripts (Inoue *et al.* 1986b, 1987). To be Rex inducible, a transcript must possess the Rex response element (RxRE) and a 5' splice donor site (Seiki *et al.* 1988). From our work and that of Seiki *et al.* (1990), it is clear that the transcript must also encode a *cis*-repressive sequence (CRS) which - most likely via interaction with some cellular factor(s) - represses posttranscriptional processing of CRS-containing transcripts. Seiki *et al.* (1990) have reported the presence of a CRS within the U5 region of the HTLV-I 5' LTR. Similarly, Black *et al.* (1991) identified a CRS element within the U5 region of the HTLV-II 5' LTR. It has been proposed that these CRS elements prevent transcripts from exiting the nucleus. Seiki *et al.* (1990) made a series of U5 and R region deletions in an HTLV-I 5'LTR-CAT expression construct. Deleting the U5 region from bp 734 or bp 701 to the end of the U5 (bp 755) resulted in no significant increase in CAT activity. Northern blot analysis of CAT mRNA showed that following these two deletions, CAT mRNA was not observed in the cytoplasm. A larger deletion from bp 620 to bp 755 resulted in a very significant increase in CAT expression and cytoplasmic CAT mRNA. Deletion from bp 585 to bp 755 resulted in no further increase in CAT expression. Deleting further upstream than bp 585 led to a major reduction in CAT expression. This is because the R region enhances transcription in the context of the HTLV-I LTR promoter (Takebe *et al.* 1988). As a result of these experiments, it was concluded that a CRS existed between bp 620 and bp 701. The appearance, shown by Northern blots, of CAT mRNA in the cytoplasm upon CRS deletion suggested that CRS-containing transcripts are trapped in the nucleus. We have shown that specific deletion of this CRS did not result in the complete removal of Rex dependency (fig. 15). These CAT assay results were supported by FISH experiments which showed that CRS removal led only to a partial release of CAT mRNA from the nucleus (fig. 40B & 41B). It was clear that present within one of the LTRs was a second CRS.

Brighty and Rosenberg (1994) showed that present within the same region of the HIV envelope gene as the Rev response element (RRE), is a *cis*-negative element. Removal of this element resulted in the Rev independent expression of HIV envelope and HIV viral replication. Their experiments were carried out using *Drosophila* cells in which unspliced HIV transcripts are retained in the nucleus in the absence of HIV Rev (Ivey-Hoyle and Rosenberg 1990). This agreed with the results of Huang *et al.* (1991). A CRS within the HIV RRE was also reported by Rosen *et al.* (1988) and Nasioulas *et al.* (1994). The HTLV-I RxRE is encoded by the 3' LTR. We have shown that deletion of the 3' LTR leads to a very significant reduction in Rex dependency, indicating that a

negative element had been removed (fig. 32). Deletion of both the 3' LTR and the 5' CRS resulted in a complete release from Rex dependency (fig. 32).

Narrowing the search down, we showed that a significant reduction in Rex dependency resulted from deletion of the RxRE (fig. 34 & 35). Transcripts lacking the RxRE were, however, still more Rex dependent than those lacking the entire 3' LTR, indicating that the downstream negative element (DNE) must extend into the U3 region. Insertion of the RxRE into plasmids lacking the 3' LTR resulted in a significant elevation of Rex dependency (fig. 37). Insertion of the HIV RRE was seen in some experiments to have no effect on the construct's Rev dependency (Chang and Sharp 1989). The authors explained this by suggesting that the CRS function of the RRE was context dependent. However, Nasioulas *et al.* (1994) demonstrated that RRE insertion did lead to an increase in the Rev dependency, even outside the context of the *env* transcript (Zolotukhin *et al.* 1994a). Analysis of fluorescence *in-situ* hybridization (FISH) experiments showed that removal of the RxRE had a similar effect to removal of the 5' CRS, namely a shift in CAT mRNA localization from the nucleus to the cytoplasm (fig. 40/41D & E). Insertion of the RxRE into a 3' LTR deleted construct (510) resulted in a significant shift back towards a nuclear localization (fig. 40/41G). In FISH experiments, as in CAT assays, complete removal of Rex dependence was only observed when both the 5' CRS and the complete DNE were removed (fig.40/41C). Brighty and Rosenberg (1994) showed via Northern blots of nuclear and cytoplasmic mRNA, that the RRE-containing *env* gp160 RNA was retained in the nucleus in the absence of Rev. Also they showed that Rev function did not depend upon the presence of HIV splice sites. Nasioulas *et al.* (1994) also demonstrated that HIV splice sites were not required for Rev function. However, because they extracted total RNA, they were not able to show that the RRE CRS was functioning via nuclear retention. Nasioulas *et al.* (1994) demonstrated with their discovery of a second CRS within the HIV *env* transcript, that CRS elements could be species specific. This new CRS, situated within the gp120 region of the *env* transcript, was functional in human cell lines but not in *Drosophila* cells. We showed that the HTLV-I DNE functioned in the human Hela and Jurkat T cell lines as well as the simian COS 7 cell line (fig. 38).

A bone of contention between HIV researchers is the significance of splicing as part of Rev's function. In vivo, Rev expression undoubtedly results in an increased ratio of unspliced to spliced viral transcripts. However, Rev binding has also been linked to subsequent processing steps such as nucleocytoplasmic transport, stabilization of cytoplasmic transcripts and enhanced ribosomal loading. It has been suggested by two research groups that active splicing or recognition of splicing signals is essential for Rev function (Chang and Sharp 1989; Lu *et al.* 1990; Hammarskjöld *et al.* 1991, 1994). Chang and Sharp (1989) used a β -globin-RRE fusion and reported that mutation of both 5' and 3' splice sites resulted in attenuation of Rev function. Hammarskjöld *et al.* (1991) used a β -globin 5' splice site - HIV *env* construct which contained the *env* 3' splice site. They reported that mutation of the 5' - but not the 3'- splice signal resulted in the attenuation of Rev

function. Brighty and Rosenberg (1994) suggested that the apparent lack of visible Rev function upon mutation of the β -globin splice sites was due to decreased transcript stability. Chang and Sharp (1989) cloned the RRE into the second intron of the rabbit β -globin gene. They made point mutations of either the 5' or 3' splice sites, or both. Constructs were transfected together with a Rev expression plasmid into COS cells. Nuclear and cytoplasmic RNA was extracted and analysed using nuclease S1 mapping. Rev function was observed as a cytoplasmic accumulation of unspliced RNA. This was observed for the non-deleted and the single splice site deleted constructs. Unspliced RNA from constructs having both splice sites mutated was seen to accumulate in the cytoplasm, independent of Rev function. Rev expression resulted in no further enhancement. The authors concluded that recognition of splice sites by the splicing machinery is required for Rev function. Brighty and Rosenberg (1994) point out that in the experiments of Chang and Sharp (1989), the level of RNA was significantly reduced following splice site mutation and that the result of splice site mutation was the destabilization of β -globin transcripts.

It has been reported that expression of β -globin is highly dependent upon its mRNA being actively spliced, allowing it to accumulate in the cytoplasm (Hamer and Leder (1979); Hamer *et al.* (1979). As both groups used the β -globin splice donor site, it could be that the observed dependence of Rev upon splicing signals was due to the inherent instability of β -globin intron-containing mRNAs.

Brighty and Rosenberg (1994) showed that mutation of the HIV 5' and 3' (*env*) splice sites did not remove Rev responsiveness. In addition, many other groups have reported that neither active splicing nor recognition of splice sites is essential for Rev's function in promoting the accumulation of incompletely spliced mRNAs in the cytoplasm (Felber *et al.* 1989; Malim *et al.* 1989; Emerman *et al.* 1989; Knight *et al.* 1987; Rosen *et al.* 1988). Taken together it seems more likely that the most significant function of HIV Rev and HTLV Rex is in overcoming the effects of cis negative elements which either retain transcripts in the nucleus (CRS) or destabilize the transcript once in the cytoplasm (instability regions). It is still possible, however, that interaction of a 5' splice site-containing transcript with the splicing machinery enhances the nuclear retention effect. Our FISH results show that the main function of HTLV-I Rex is the nuclear export of CRS-containing transcripts (fig. 24 & 40b).

One significant difference between the CRS elements discovered in HIV and HTLV viruses is that the HIV CRS elements are located within the coding region, whilst the HTLV CRS elements are within the non-translated regions. As well as the two CRS elements shown to exist in the gp41 and gp120 regions of HIV *env* (Brighty and Rosenberg, 1994; Nasioulas *et al.* 1994) there have also been reports of an RNA destabilizing sequence (INS-1) within the HIV *gag* gene (Schwartz *et al.* 1992a). This element was found to be AU rich, a feature that mRNAs of short half life have in common and which may lead to their preferential deadenylation and nuclear degradation. Efficient HIV Gag expression could be obtained either upon addition of Rev or through introducing specific

mutations within the destabilizing region (Schwartz *et al.* 1992b). As the HIV *env* gene has already been shown to contain two separate CRS elements, which would be present not only in *env* transcripts but also within *gag-pol* transcripts, it would seem unnecessary for the *gag* gene to encode its own. It is possible that the *gag* CRS (INS-1), which acts to destabilize the RNA acts as an extra control mechanism to prevent "leaky" expression of structural genes before the main wave of Tat/Rev initiated gene expression. Such a mechanism could allow the virus to "hide" more effectively from the host's immune system. It is interesting that Rev is able to overcome not only the nuclear retention effect of the HIV *env* CRS elements but also the RNA destabilizing effect of the HIV *gag* INS element. The HTLV-I 5' CRS appears to function in the same way as the HIV *env* CRS elements, preventing unspliced transcripts from leaving the nucleus. The 3' DNE, also shown to inhibit nuclear export of RNA, could be functioning as an extra control mechanism preventing the leaky expression of all HTLV-I genes, including the regulatory genes *tax* and *rex*. This would be expected to result in very low basal expression of the *tax* gene in HTLV-I infected patients relative to the HIV *tat* gene in HIV infected patients. Indeed it has been reported by Nerenberg *et al.* (1987) that levels of Tax protein expression directed by an HTLV-I based vector system were unexpectedly low. This may be due to the apoptotic effects of Tax (Chlichlia *et al.* 1995) but also to the fact that the 3' LTR was used. The presence of a *cis* repressive element within the *tax* and *rex* transcripts could partly explain the difference in latency period between HIV and HTLV.

Cis negative elements have not only been identified in retroviruses. The L1 transcript of Human Papilloma Virus has been shown to contain two separate RNA "instability" regions (Tan and Schwartz 1995; Kennedy *et al.* 1994). These were shown not to inhibit the transport of transcripts to the cytoplasm but instead to prevent efficient translation once there. L1 expression could be restored by inserting the HIV RRE and expressing Rev. The "instability" regions were shown to have AU contents of approximately 59%, similar to the 61.5 % reported by Schwartz *et al.* 1992a, 1992b) for the HIV *gag* INS-1. The authors speculate that AU richness may lead to a transcript being bound by a cellular factor and transported to a cytoplasmic compartment in which translation is prevented.

A Rex response element (RxRE) encoded by the 5' LTR

It is well accepted that Rex functions via binding to the RxRE encoded by the 3' LTR (Seiki *et al.* 1988). The sequence and structural specificities of the Rex binding site have been delineated via mutagenesis analysis (Toyoshima *et al.* 1990, Grassmann *et al.* 1991). A most unusual observation was made when investigating the effect of Rex upon transcripts lacking the 3' RxRE. Rex was repeatedly observed to enhance their CAT expressions!

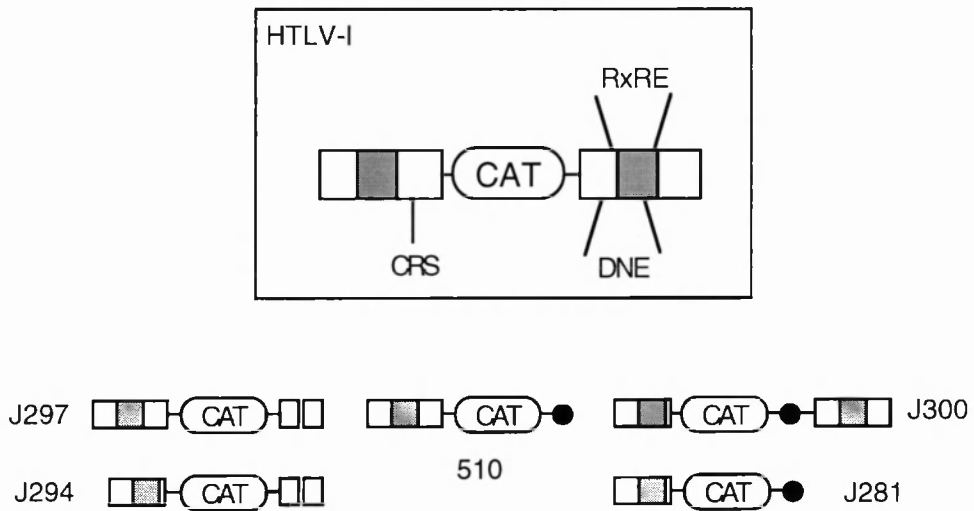


Figure 42. 3' RxRE deleted constructs showing different levels of Rex inducibility.

NB. The exact boundaries of the DNE within the 3'LTR are not known.

Constructs lacking the 3' RxRE but containing either part of the DNE (J297) or both this and the 5' CRS (J294) were seen, reproducibly to be 2-3 and 8-10 fold Rex inducible, respectively (fig. 34 & 35). 2-3 fold Rex inductions were also observed from constructs (510 and J301) possessing the full 5' LTR but lacking the entire 3' LTR (fig. 30,32, 34 & 35). Only constructs lacking both the 5' CRS and the entire DNE (J281, J300) were Rex non-inducible (fig. 32, 34 & 35). Initially it was suspected that a low level of incorrect termination of transcription at the 5' LTR of our circular plasmids was responsible for this apparent artifact. However, Rex inductions were still observed after experiments were repeated using plasmids linearized just upstream of the 5' LTR, leading us to believe that this was not an artifact (results not shown). The only reasonable explanation for these data is that Rex is able, albeit with reduced efficiency, to bind to the Rex binding stem loop encoded by the 5' LTR.

In HTLV-I the 3' RxRE is formed and stabilized by the annealing of a stretch of nucleotides just downstream of the polyadenylation signal with a stretch just upstream of the polyadenylation site. This is referred to as stem A. Because the RNA cap site (nt 306) is within the R region of the 5' LTR, the first stretch of nucleotides contributing to stem A (nt 337 to 362) does not exist, and so a significant portion of the RxRE is unable to form. However, the Rex binding stem loop is formed by nucleotides 506 to 550 and is thus present not only within the full length (755bp) 5' LTR but also within the CRS-deleted LTR₆₂₈, the CRS beginning at nucleotide 620. The putative 5' LTR Rex binding motif could still be stabilized by base pairing from nucleotide 423 to 466.

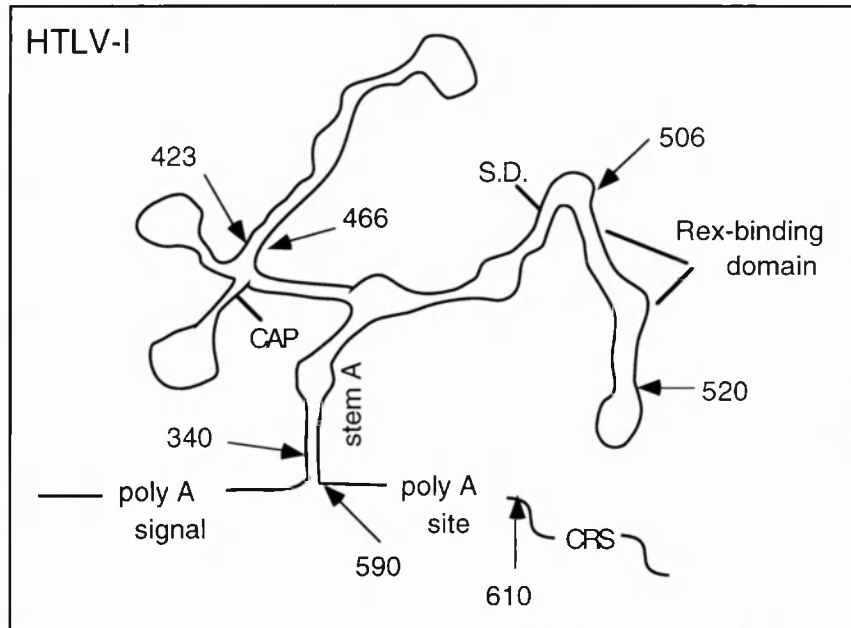


Figure 43. Proposed secondary structure of RNA encoded by the HTLV-I 3' LTR.

In HTLV-II, two RxREs have been identified. The first is present at the R/U5 boundary of the 5' LTR and the second at the U3/R boundary of the 3' LTR. It has been shown that a CRS is present just downstream of the 5' RxRE of HTLV-II and that this RxRE is sufficient for Rex function (Black *et al.* 1991). The 3' RxRE appears to be redundant. The HTLV-II 5' RxRE is more stable than the proposed HTLV-I 5' RxRE because the HTLV-II cap site is upstream of the stabilizing stem A. The HTLV-II CRS element identified within the 5' LTR is not repeated in the RNA encoded by the 3' LTR as it is downstream of the polyadenylation site. This is also the case for the HTLV-I CRS.

Remembering that HTLV-I and HTLV-II are very closely related, it would seem likely that a common HTLV ancestor was able to bind Rex at both RxREs equally well. HTLV-II then evolved towards a greater dependence upon Rex binding at the 5' RxRE, perhaps through a requirement or preference for a more direct interaction with the 5' splice donor site. HTLV-I, on the other hand evolved towards a greater dependence upon Rex binding at the 3' RxRE. An important consequence of this variation is that HTLV-I Rex binding tends to stabilize all three structural genes' expressions whereas HTLV-II Rex binding must preferentially stabilize the *gag-pol* transcript. Singly spliced *env* transcripts no longer have the 5' RxRE and can thus only be Rex induced via binding to the 3' RxRE. An intermediate situation exists in HIV, where Rev is able to bind both the unspliced and singly spliced transcripts, the RRE being present within the *env* coding region.

The presence - *in cis* - of a sequence derived from the MLV *gag* gene is sufficient to remove the Rex dependency of HTLV-I LTR transcribed mRNAs.

Seiki *et al.* (1990) reported that HTLV-I LTR transcribed mRNAs are retained in the nucleus due to the presence of a *cis* repressive sequence (CRS) in the 5' non-translated region. Our own work - using Fluorescence *in-situ* Hybridization to visualize specific mRNAs transcribed by the HTLV-I LTR - showed directly that this is indeed the case. We observed that HTLV-I constructs containing the MLV extended packaging sequence (ψ^+) did allow expression of a CAT reporter gene in the absence of Rex expression (fig. 12 & 13). Removal of the ψ^+ resulted in the return of complete Rex dependency (fig. 15). Analysis of the ψ^+ when inserted in the reverse orientation was not possible due to the presence of multiple translation start / stop signals. Instead, the ψ^+ was divided up into four different regions, each then being re-inserted in the positive orientation. CAT assays using these constructs showed that the observed ψ^+ effect was sequence specific and not merely dependent upon the length of the DNA fragment inserted (fig. 19). A specific ψ^+ effect was localized within the last 312bp of the ψ^+ sequence. This region corresponds to the p15 coding region of the MLV *gag* gene (fig. 27).

Because the observed ψ^+ effect was similar in nature to the result obtained upon removal of the 5' CRS, it was initially suspected that the ψ 312 fragment may contain a splice acceptor site. Splicing into such a site from the R region splice donor would have resulted in removal of the 5' CRS and more efficient expression through having had an intron spliced out. Although there are no known splice acceptor signals within the ψ 312 fragment, it was possible that a cryptic site was being utilized. Recently, Oshima *et al.* (1996) described cryptic MLV splicing events. These splice sites were, however, all downstream of the region corresponding to the ψ 312 fragment and were shown to be extremely weak. The fact that these weak signals were downstream further supports our idea that the ψ 312 fragment does not even contain any cryptic splice acceptor sites. Northern blot analysis showed that RNA from COS7 cells transfected with the ψ 312 containing construct was, as expected, larger than the control construct which lacked the ψ fragment (fig. 22). This again supported the idea that the observed ψ^+ effect was not due to splicing out of the 5' CRS element.

Fluorescence *in-situ* hybridization was used to determine whether the ψ 312 fragment was able to remove the nuclear retention, already seen to exist for transcripts derived from CRS-containing 5' LTRs. The presence of either the ψ 312 or complete ψ (872bp) sequence was seen to produce a very significant shift from nuclear to cytoplasmic CAT RNA localization (fig.25 & 26). CAT RNA from constructs lacking the ψ or containing the ψ 560 fragment were seen to be retained in the nucleus (fig. 25 & 26).

A common question has always been whether Rex enhances a transcript's expression by inhibiting its association with the splicing machinery, promoting its transport to the cytoplasm, stabilizing it

once in the cytoplasm or through enhancement of the translation process. We have shown that the same shift in localization of RNA to the cytoplasm is seen upon insertion of the psi 312 fragment as upon Rex enhancement of CAT expression in a construct lacking the psi fragment (fig. 24 & 25). This indicated that the major function of the psi 312 fragment in our system is to allow the Rex independent transport of CRS containing transcripts to the cytoplasm where they can then be translated.

Bray *et al.* (1994) described a similar phenomenon after inserting a fragment of the Mason Pfizer Monkey Virus (MPMV) genome into a Rev dependent, HIV *env* expression construct. The MPMV insertion resulted in the removal of Rev dependency. The authors argue that the 219 bp fragment of MPMV (bp 8022-8240) was able to enhance expression of *env* by promoting the nuclear export of *env* transcripts and thus termed it a "constitutive transport element" (CTE). They proposed that CTE recognition by some cellular factor(s) could target the RNA-protein complex to a nucleocytoplasmic transport pathway, avoiding interaction with the splicing machinery.

Zolotukhin *et al.* (1994b) showed that an element from simian retrovirus type 1 (SRV-1) was able to overcome the requirement for HIV Rev and the RRE, allowing the continuous propagation of two different HIV molecular clones. As mentioned previously, the L1 transcript of the human papilloma virus contains two instability elements (Kennedy *et al.* 1991; Tan and Schwartz 1995). It was shown that although transported to the cytoplasm, they were not translated. The SRV-1 CTE was shown to be able to overcome this inhibition, allowing expression of L1 protein (Tan *et al.* 1995). Bray *et al.* (1994) showed that the MPMV CTE and the SRV-1 CTE share 93% nucleotide composition identity. No significant identities were found between the MLV CTE and the CTE elements of MPMV and SRV-1. It would seem likely that these two D-type retroviruses utilize a cellular factor to promote the transport of their genomic CTE-containing transcripts in a similar way to HIV, HTLV and bovine leukaemia virus (BLV), which all encode their own viral posttranscriptional regulators (Rev and Rex etc).

Although Rex was no longer required for expression of psi 312 CTE containing transcripts, it did further enhance expression. The same observation was made by Bray *et al.* (1994), Rev being able to further enhance the HIV *env* expression from the MPMV CTE containing construct. The authors suggested that Rev was still able either to stabilize the RNA or "act in concert with the MPMV element to accomplish export". The MPMV CTE was also seen to function more efficiently, relative to Rev induction alone, in simian CMT3 cells but not in human Chang liver cells, suggesting some degree of variation between different cell types. The psi 312 fragment was seen to function in human Jurkat T cells, human Hela cells and simian COS7 cells (fig. 20). However, the psi 312 effect was not compared to the Rex induction alone for all of the cell lines.

CTE - possible mechanism of action

Recent investigation of the nuclear export mechanism suggests that RNA molecules are transported into and out of the nucleus by specific transport proteins and not as "naked" RNA molecules. The model system used to examine the process of nuclear RNA export is that of HIV Rev binding to the RRE. It has been shown that neither the RNA alone nor the RNA-Rev complex is able to exit the nucleus alone. A cellular factor was required. Two-hybrid experiments performed in human and yeast systems - both using the Rev activation domain as "bait" - led to the identification of two distantly related proteins termed human hRIP and yeast RIP 1p, respectively (Fritz *et al.* 1995, Bogerd *et al.* 1995, Stutz *et al.* 1995). The interaction of Rev and RIP has been shown to depend on a nuclear export signal (NES), present within the activation domain of Rev. Similar NES motifs have been identified within nuclear proteins that are known to be exported to the cytoplasm (Fischer *et al.* 1995, Wen *et al.* 1995, Fridell *et al.* 1996).

The psi 312 CTE must therefore either directly bind a nuclear export factor or an NES containing protein which then through its NES interaction with a nuclear export factor is exported to the cytoplasm. To address this issue, three constructs were made for use in *in-vitro* transcription experiments (fig. 28). Should the radioactively labelled psi 312 RNA bind a cellular factor, this binding would be visualized in a gel mobility shift assay or in a North-western blot analysis. The RxRE and psi 201 fragment containing constructs were prepared as positive and negative controls for RNA binding.

The discovery of a CTE element within the MLV extended packaging sequence helps to explain the observed increase in packaging efficiency upon its inclusion into retroviral vectors. In this case, nucleocytoplasmic transport of the vector transcripts is upregulated which indirectly leads to increased packaging (Bender *et al.* 1987). It is thus likely that psi⁺ sequences do not directly mediate the encapsidation / packaging process.

Outlook

The development of new vector systems for use in diverse gene therapy protocols has recently been prioritized by the *NIH gene therapy review committee*. Problems such as low effective virus titer, post-integration expression loss and the absence of good inducible expression systems will need to be addressed. HTLV-I based retroviral vectors present an alternative to the murine based vector systems, offering new perspectives for future vector development.

The HTLV-I LTR, having evolved within the human host may allow a more efficient expression in human target cells. Through incorporation of the two *cis* negative elements (CRS & DNE) and use of the non-toxic Rex gene, a gene cloned between the LTRs could be inducibly expressed, control being exerted at the posttranscriptional level. It has also been demonstrated that HTLV-I based vectors allow the double copy modification through insertion of an expression cassette into the 3'

LTR U3 region (Aat II site). For more efficient expression of a gene cloned between the LTRs in both standard and DC vector designs, a 3' splice acceptor site should be inserted immediately downstream of the packaging signal to allow the genes expression from a spliced transcript.

The use of constitutive transport elements (CTE) *in cis* to increase the efficiency of nuclear export will allow a further enhancement of vector gene expression. This may be of particular use when expressing transcripts which are unstable and readily degraded in the nucleus. As observed for the SRV-1 CTE, it is possible not only to enhance nucleocytoplasmic transport but also to enhance the expression of AU rich INS instability elements such as HIV-2 vif, c-myc, c-fos, GM-CSF (Jones *et al.* 1987; Rabbitts *et al.* 1985; Shaw *et al.* 1986; Shyu *et al.* 1989; Triesman 1985; Wilson and Triesman 1988).

Before such viruses could be used *in-vivo*, they would need to be rendered Tax independent. Replacement of the Tax response elements in the U3 region of the 3' LTR by a house keeping enhancer would render viral transcription Tax independent, following reverse transcription and integration into the host's genome. An added benefit from this modification - which would result in a reduced nucleotide identity between the two LTRs - is that general (ie. recA independent) recombination events in E.Coli would be less likely.

In summary, the development of HTLV-I based retroviral expression constructs has provided important insight into the manner in which retroviruses regulate the coordinate expression of their genes. Understanding the involvement of viral *cis* positive and negative sequences enables us to take advantage of their unique properties in designing new, more efficient and more flexible viral vectors.

Ac	Acetate
ADA	Adenosine Deaminase
AIDS	Acquired Immune Deficiency Syndrome
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid
BIS	N,N'-Methylene-bis-acrylamide
BSA	Bovine Serum Albumin
CAS	<i>Cis</i> -Activating Sequence
CAT	Chloramphenicol Acetyltransferase
cDNA	Copy DNA
Co.A	Coenzyme A
CIP	Calf Intestine Phosphatase
CREB	cAMP response element binding
CRS	<i>Cis</i> -Repressive Sequence
CTE	Constitutive Transport Element
CTP	Cytosine Triphosphate
DEAE	Diethylaminoethyl-
DEPC	Diethylpyrocarbonate
dH ₂ O	distilled water
DIG	Digoxoginin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxy-ribose Nucleic Acid
dNTP	Deoxy-Nucleotide Triphosphate
DTT	Dithiothreitol
E.Coli	<i>Escherichia Coli</i>
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid
EtBr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FDG	Fluorescene di-β-D-Galactopyranoside
FISH	Fluorescence in-situ Hybridization

GDP	Guanosine Diphosphate
GTE	Glucose / Tris / EDTA
GTP	Guanosine Triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N-2'-ethanosulphonic acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
IRES	Internal Ribosome Entry Signal
HTLV	Human T cell Leukaemia Virus
LB	Luria Bertani
LSM	Laser Scanning Microscope
MOPS	3-(N-Morpholino) propanesulphonic acid
mRNA	messenger RNA
MuLV (MLV)	Murine Leukaemia Virus
NES	Nuclear Export Signal
NF	Nuclear Factor
NIH3t3	National Institutes of Health 3t3 Fibroblast cell line
NLS	Nuclear Localization Signal
NTR	Non-Translated Region
OD	Optical Density
Oligo	Oligonucleotide
ORI	Origin of Replication
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PETG	Phenethylthio- β -D-Galactopyranoside
PI	Propidium Iodide
prep.	preparation
RE	Restriction Endonuclease (enzyme)
RNP	Ribonucleoprotein
RRE	Rev Response Element
RxRE	Rex Response Element
RNA	Ribonucleic Acid
RT	Room Temperature or Reverse Transcriptase
RT-PCR	Reverse Transcriptase PCR

SDM	Site Directed Mutagenesis
SDS	Sodium Dodecylsulphate
SSC	Salt Saturated Citrate
SV 40	Simian Virus 40
TAE	Tris / Acetate / EDTA
Taq	<i>Thermophilus</i> Aquaticus
TBE	Tris / Borate / EDTA
TBS	Tris Buffered Saline
TE	Tris / EDTA
TELT	Tris / EDTA / Lithium Chloride / Triton X-100
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLC	Thin Layer Chromatography
Tris	Tris-(hydroxymethyl)-aminomethane
TTP	Thymidine Triphosphate
UTP	Uridine Triphosphate
v/v	Volume / Volume
w/v	Weight / Volume
X-Gal	5-Bromo-4 Chloro-3-indolyl- β -D-galactopyranoside

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